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As a result of shortage of paper the annual volume of Antonie van Leeuwenhoek may contain merely $\frac{2}{3}$ of its usual number of pages.

Wegen Papierknappheit sind wir gezwungen, die übliche Seitenzahl des Jahresbandes von Antonie van Leeuwenhoek auf $\frac{2}{3}$ herabzusetzen.

A cause de disette en papier le volume annuel de Antonie van Leeuwenhoek n'ose surmonter $\frac{2}{3}$ du nombre habituel de pages.

PROFESSOR DR JOHANNA WESTERDIJK

1917–1942

Am 10ten Februar 1917 wurde Dr JOHANNA WESTERDIJK als ausserordentlicher Professor der Phytopathologie an die Universität Utrecht berufen. Dieses Ereignis bedeutete damals ein doppeltes Novum: die Einstellung eines neuen universitären Lehrkanzels und das erste Auftreten einer Frau als Professor in den Niederlanden. Die seitdem vergangen 25 Jahre haben die Bedeutung beider Ereignisse genügsam erwiesen. Die Schriftleitung findet denn auch hierin Gründe, das Jubiläum dieses Mitgliedes der Redaktionskommission zu gedenken.

Da Professor WESTERDIJK ihre wissenschaftliche Laufbahn sehr jung angefangen hat, konnte schon im Jahre 1931 das silberne Jubiläum ihrer Ernennung als Direktorin des Phytopathologischen Laboratoriums WILLIE COMMELIN SCHOLTEN gefeiert werden. In dem bei dieser Gelegenheit erschienenen speziellen Heft des „Vakblad voor Biologen“ (5) hat Prof. F. A. F. C. WENT eine wohldokumentierte Beschreibung ihres Lebenslaufes und ihrer wissenschaftlichen Leistungen bis 1931 veröffentlicht; ferner hat Herr A. VAN LUYK eine Liste der von 1906–1931 erschienenen Untersuchungen im Laboratorium WILLIE COMMELIN SCHOLTEN zusammengestellt (3). Die Schriftleitung ist der Meinung, dass Wiederholungen unerwünscht sind und demgemäss wird unser Artikel sich auf die Professur der Jubilarin beschränken.

Schon vor der Besetzung des Lehrstuhles hatte die Utrechter Fakultät, und namentlich Professor WENT, sich lange bemüht um zur Ein-



stellung eines Lehrkanzels für die Pflanzenpathologie zu geraten. Das Gebiet der Pflanzenpathologie hatte sich eben stark ausgedehnt und an diejenigen, die sich phytopathologischer Arbeit widmeten, wurden sehr spezielle Anforderungen gestellt. Für Europa war der Utrechter Universitäre Lehrstuhl der einzige seiner Art; in Nord-Amerika jedoch machte die Pflanzenpathologie schon seit längerer Zeit Teil des Lehrplanes vieler Universitäten aus und erweckte grösseres Interesse.

DR JOHANNA WESTERDIJK war unbedingt die rechte Person zur Erfüllung dieses Amtes. Nicht nur war sie damals schon während 11 Jahren Direktorin des Laboratoriums WILLIE COMMELIN SCHOLTEN, aber auch war sie auf eine längere Reise durch Niederländisch Indien, Japan und die Vereinigten Staaten in persönlichen Kontakt mit den voranstehenden Fachgenossen in jenen Ländern gekommen und hatte die Aufgaben, vor deren Lösung diese gestellt waren, kennen gelernt. Dazu hatte sie die wichtigsten wissenschaftlichen Fachzentren in Europa besucht. Ihre eigene wissenschaftliche Arbeit hatte sie in Berührung gebracht mit Landwirtschaft und Gartenbau und sie verstand sich ausgezeichnet auf den Umgang mit Praktikern. Keiner würde besser als sie das Interesse der Studenten auf praktische Fragen zu lenken wissen und ihnen die Lust zur Lösung solcher Fragen einflössen.

Als Gegenstand ihrer Inauguralrede wählte sie: Nieuwe wegen der Phytopathologie (Neue Wege der Phytopathologie). Sonnenklar wurden hier die verschiedenen Aspekte des neuen Lehrfaches erhellt und auf die Berührungspunkte mit älteren Lehrfächern gewiesen. Scharfe Richtlinien wurden gezogen nach denen die wissenschaftliche Untersuchungen sich zu bewegen haben. Sehr nachdrücklich betonte sie, wie wünschenswert bei der Ausübung gerade dieses Faches der internationale Kontakt und ein offener Blick über die eigenen Landesgrenzen hinaus sind: öfters sei dies ja notwendig um zur Ausarbeitung zuverlässiger Bekämpfungsmethoden zu geraten. Als die Umstände dies noch erlaubten, haben viele ihrer Schüler diesem Rat Folge geleistet und sind weit ausgeflogen.

In den ersten Jahren ihrer Professur hatte sie mit Mangel an einem Versuchsgarten und Experimentierraum zu kämpfen. Das Laboratorium WILLIE COMMELIN SCHOLTEN in einem städtischen Haus in Amsterdam mit schmalen dunklen Garten bot ja nicht die Gelegenheit dazu. Eine grosse Verbesserung trat ein, als der Cantonspark in Baarn von der Familie JANSSEN zum Hortus der Utrechter Universität bestimmt wurde. Der frühere Obstgarten und ein gutes Gewächshaus wurden zur Verfügung von Professor WESTERDIJK gestellt. Im Anschluss

hieran siedelte das Laboratorium nach Baarn über, wo ein altes geräumiges Haus „Villa Java“ in grossem Garten bezogen wurde. Und hier in Baarn fand Professor WESTERDIJK die Umstände, die es ihr gestatteten, den Unterricht in der Phytopathologie nach eigenen Einsichten und Wünschen einzurichten. Nur die wöchentlichen Vorlesungen fanden noch in Utrecht statt. Die praktische Arbeit der Studenten wurde vollends nach Baarn verlegt, wo im Frühling und Sommer, wenn Pflanzenkrankheiten im freien zu beobachten und zu studieren sind, die Studentenpraktika ohne Zwischenpausen einander folgen.

Es ist nicht möglich, die 25-jährige Professur zu besprechen, ohne auf die sehr besonderen Verhältnissen in Baarn bezugzunehmen. Die Umgebung bietet ja die Gelegenheit zur Pflanzenbeobachtung und zur Züchtung der erfreulich gewachsenen „Kontrollen“, worauf Professor WESTERDIJK besonderen Wert legt. In Villa „Java“ ist ausserdem das „Centraal Bureau voor Schimmelcultures“ untergebracht als besonderes Institut, dessen Leitung von Professor WESTERDIJK geführt wird. Das „Centraal Bureau“ verleiht dem Institut einen internationalen Charakter: beim normalen Verkehr besuchen viele Ausländer diese stets heranwachsende Sammlung von Schimmelpilzen und gibt es einen regen brieflichen Verkehr über wissenschaftliche Fragen. In der Sammlung ist ein mykologisches Vergleichsmaterial vorhanden wie nirgends sonst. Den Studenten wird demnach eine einzigartige Gelegenheit geboten sich in die Geheimnisse der Mykologie einzuarbeiten und erfahrene Mykologen können in schwierigen Fällen Hilfe leisten. Während vieler Jahre war der erste Assistent, Herr A. VAN LUYK, Mitarbeiter bei der Lösung aller Schwierigkeiten. In seinen letzten amtlichen Jahren hat er wichtige Untersuchungsarbeit geleistet über den antagonistischen Einfluss von Mikroorganismen auf den Virulenzgrad (4) und in einigen der späteren Doktorarbeiten (TEN HOUTEN, JAARVELD) ist dieser Gegenstand näher studiert worden.

Das Laboratorium WILLIE COMMELIN SCHOLTEN wurde alsbald das Zentrum des phytopathologischen Studiums aller universitären Studenten in den Niederlanden. Nachdem 1930 die Universität von Amsterdam Professor WESTERDIJK gleichfalls als ausserordentlicher Professor an sich verbunden hatte, hat die Anzahl Studenten aus Amsterdam sich noch vermehrt. In ihrer zweiten Antrittsrede „De groei der Phytopathologie“ (Das Wachstum der Phytopathologie) gibt sie einen meisterhaften Überblick über den damaligen Stand ihres Faches. Nur der Anteil, den sie selber zu diesem Wachstum beigetragen hat, namentlich durch die Ausbildung ihrer Schüler wird nicht erwähnt. Wohl zeigte die

Rede sehr klar mit welcher Begeisterung Professor WESTERDIJK ihre eigentliche Unterrichtsaufgabe erfüllt. Am besten lassen wir sie selbst ans Wort kommen, wie sie sich damals an die Studenten richtete:

„Sie sehen wie fehlerhaft Tradition oft wirkt: diese fordert ja, dass der neuberufene Professor an letzter Stelle Ihnen zuspricht, während Sie doch die direkte Ursache meines heutigen Hierstehens sind. Sie glauben mir doch, dass ich stolz bin, dass Sie den Antrieb zu diesem Ereignis gegeben haben? Was aus natürlichem Drang herauswächst, hat die beste Aussicht auf Erfolg. Ich brauche Sie weder zu bemitleidigen wegen einer Zunahme der Vorlesungen, noch „die Hoffnung auszusprechen“, dass wir angenehm zusammenarbeiten werden, weil ich schon längst weiss, dass zwischen ihnen und mir eine gesunde Zusammenarbeit besteht. Viele von Ihnen habe ich schon in die praktische phytopathologische Arbeit eingeführt, ausgezeichnete Assistenten habe ich aus Ihrer Mitte erhalten; Wehklagen über den Abstand Amsterdam–Baarn haben mich nicht erreicht. Sie haben ein Recht darauf, dass auch ich den Abstand Baarn–Amsterdam nicht zählen werde. Und ich spüre den Rhythmus eines fröhlichen Marsches, wenn ich an die Reihen begeisteter junger Menschen aus Ihrer Mitte denke, die ausziehen werden um die Phytopathologie zu vollem Wachstum zu bringen“.

Die wissenschaftliche Fragen, die Professor WESTERDIJK persönlich bevorzugt, sind die der Baumkrankheiten. Der langen Wachstumszeit der Versuchspflanzen wegen, bieten Versuche mit Bäumen besondere Schwierigkeiten und sind meistens nur langwierigen Untersuchungen zugänglich. Trotzdem hat die erste Doktorarbeit, die unter ihrer Leitung gemacht wurde (MARIE B. SCHWARZ, Das Zweigsterben der Ulmen, Trauerweiden und Pfirsichbäume) schon in kurzer Zeit den Beweis geliefert, daß *Graphium ulmi* der Erreger der Ulmenkrankheit sei. In folgenden Jahren ist die Ulmenkrankheit ein Untersuchungsobjekt geblieben und zusammen mit Dr BUISMAN hat Professor WESTERDIJK eine Monografie „De iepenziekte“ (6) veröffentlicht. Auch die Untersuchungsarbeit der gegenwärtigen ersten Assistentin, Dr H. C. KONING (2) über den Krebs der Pappel — eine Krankheit, welche wohl in erster Linie von einem Bakterium verursacht wird — hat ihr grosses Interesse.

In wissenschaftlichen nationalen oder internationalen Kreisen sind ihre Vorträge, die immer schön aufgebaut und glänzend vorgetragen werden, sehr beliebt. Auch für die Biologenreise nach Süd-Afrika im Jahre 1938 trifft das zu und sie hat sehr viel zum guten Erfolg dieser Reise beigetragen.

Der Schwerpunkt ihrer wissenschaftlichen Arbeit liegt jedoch in der wissenschaftlichen Ausbildung, die sie ihren zahlreichen Schülern zukommen lässt. In der vergangenen 25-jährigen Periode sind 41 Doktor Arbeiten unter ihrer Leitung vollendet und weitere 7 sind noch im Bearbeitung. Aus der diesem Artikel beigefügten Liste dieser Arbeiten ergibt sich, auf wie verschiedenen Gebieten die Studenten ihre Themata gewählt haben. Tatsächlich gibt es nur wenige Gebiete der Pathologie, die hier nicht einmal behandelt worden sind; solche sind dann meistens Gebiete, die nur mittels einer passenden speziellen Ausstattung behandelt werden können.

Unter den Doktorarbeiten trifft man solche, die eine spezielle Krankheit zum Gegenstand haben (u.a. PFÄLTZER, TIDLENS, LINDEYER, DE BUSSY), rein mykologische (BERKHOUT, BOLLE), Analysen des Sammelbegriffes Wurzelfäule (ROOLENBURG, BUISMAN, MEURS, VAN EEK), anatomische Bearbeitung der pathologischen Symptome (LIERNUR, KERLING, VAN VLIET, KLINKENBERG), physiologische Untersuchungen der Wirtspflanze (BROEKHUIZEN, PLANTENGA), Untersuchungen der Nahrungsphysiologie der Parasiten (BUNSCHOTEN, WEHLBURG, REITSMA, VAN DER VEEN), aktive Immunisation (DUYFJES), Wirkung der Fungiciden (HARTSUIJKER).

Eine bestimmte Krankheitsgruppe, die in Baarn mit grossem Erfolg bearbeitet worden ist, ist die der Mangelkrankheiten. Nachdem einige Jahre vorher von WARINGTON die Unentbehrlichkeit des Bors für eine normale Entwicklung von *Vicia faba* angezeigt worden war, gelang es MARGARETHA MES Bormangel Symptome bei in borfreier Nährlösung gezüchtetem Tabak auszulösen. Von Professor WESTERDIJK wurde erkannt, dass diese Symptome mit denen der „Topziekte“ in Deli, einer Krankheit, welche dort auf bestimmtem Böden die Tabakskultur gefährdet, übereinstimmten. Düngungsversuche in Deli haben erwiesen, dass tatsächlich mittels einer Borgabe diese Krankheit vorgebeugt werden kann. Zum ersten Mal war gezeigt worden, dass im Bormangel die Ursache von rätselhaften Bodenkrankheiten liegen könnte. In 1931 gelang es Dr BRANDENBURG aus Bonn in Baarn in borfreie Lösungen die Symptome der Herzfäule bei Rüben zu erhalten (1). Nach jahrelangem Suchen in vielerlei Richtung war die Frage nach der Ursache der gefürchteten Herzfäule endgültig gelöst und der Weg zum Studium von Bodenkrankheiten vieler Gewächse in allen Teilen der Welt war geöffnet. Landwirtschaftlich ist Bormangel sogar nahezu Modesache geworden.

Es ist wohl angebracht, in einer Besprechung der Arbeit von

JOHANNA WESTERDIJK als Professor der Arbeit ihrer Schüler viel Raum zu lassen. Liegt ihr doch die wissenschaftliche und am meisten die menschliche Bildung ihrer Schüler sehr nah am Herzen. Selber als Mensch allseitig ausgebildet, ebenso sehr zu Hause in der Welt der Musik wie in ihrem Fachgebiet, kann sie mit Menschen sehr verschiedener Art Berührungspunkte finden. Ich habe sie einmal sagen hören „tatsächlich sei sie ein richtiger Schulfuchs“. Dies trifft nun nicht zu: sie ist eben Erzieherin im grossen Stil. Sie hat die Gabe aus Menschen Seiten zu Vorschein zu zaubern, denen diese sich selber nicht bewusst waren. Wenige ihrer Studenten sind nicht menschlich gefördert und unter ihrem Einfluss persönlich entwickelt.

Die erste Niederländische Frau als Professor erfüllt nicht nur glänzend ihre wissenschaftliche Aufgabe, aber ihre menschliche Leistung ist ihrer hohen wissenschaftlichen ebenbürtig.

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Wageningen, 19. Januar 1942.

MARIE P. LÖHNIS.

Liste der Doktorarbeiten 1917–1942

1. MARIE B. SCHWARZ, Das Zweigsterben der Ulmen, Trauerweiden und Pflirsichbäume. Utrecht 1922.
2. MARIE P. LÖHNIS, Onderzoek over *Phytophthora infestans* (Mont.) de By op de aardappelplant. Utrecht 1922.
3. CHRISTINE M. BERKHOUT, De schimmelgeslachten *Monilia*, *Oidium*, *Oospora* en *Torula*. Utrecht 1923.
4. PIERRETTE C. BOLLE, Die durch Schwärzepilze (*Phaeodictyae*) erzeugten Pflanzenkrankheiten. Utrecht 1924.
5. K. SIMON THOMAS, Onderzoekingen over *Rhizoctonia*. Utrecht 1925.
6. CATHARINA M. DOYER, Untersuchungen über die sogenannten Pestalozzia-Krankheiten und die Gattung *Pestalozzia* de Not. Utrecht 1925.

7. J. M. Roodenburg, Zuurstofgebrek in den grond in verband met wortelrot. Utrecht 1927.
8. Christine J. Buisman, Root rots caused by *Phycomycetes*. Utrecht 1927.
9. J. C. s' Jacob, Anorganische beschadigingen bij *Pisum sativum* L. en *Phaseolus vulgaris* L. Utrecht 1927.
10. J. P. Karthaus, Het afsterven van stengels en knoppen bij de roode framboos. Utrecht 1927.
11. A. Pfältzer, Het vrucht- en bladvuur van de komkommer *Cladosporium cucumerinum* Ell. et Arth. en *Corynespora melonis* (Cooke) Lindau. Utrecht 1927.
12. A. G. M. Liernur, Hexenbesen, ihre Morphologie, Anatomie und Entstehung. Utrecht 1927.
13. Louise C. P. Kerling, De anatomische bouw van bladvlekken. Utrecht 1928.
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15. S. Broekhuizen Jr., Wondreaksies van hout. Het ontstaan van thyllen en wondgom, in het bijzonder in verband met de iepenziekte. Utrecht 1929.
16. Huguette C. C. A. A. Vos, De invloed van *Pseudococcus citri* (Risso) Fern. op de plant. Utrecht 1930.
17. R. van der Veen, Onderzoekingen over tracheomycosen. Utrecht 1930.
18. Margaretha G. Mes, Fisiologische Siektesimptome van tabak. Utrecht 1930.
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20. A. Brinkman, De roodneuzenziekte van *Phaseolus vulgaris* L. veroorzaakt door *Pleospora herbarum*. Utrecht 1931.
21. F. H. Feekes, Onderzoekingen over schimmelziekten van bolgewassen. Utrecht 1931.
22. W. F. van Hell, Onderzoekingen over ziekten van lelies. Utrecht 1931.

23. C. WEHLBURG, Onderzoekingen over erwtenanthracnosen. Utrecht 1932.
24. J. REITSMA, Studien über *Armillaria mellea* (Vahl) Quél. Utrecht 1932.
25. HERMA PLANTENGA, Pathologische veranderingen in het phloem. Utrecht 1932.
26. EGBERTA J. LINDEYER, De bacterieziekte van den wilg veroorzaakt door *Pseudomonas saliciperda* n.sp. Amsterdam 1932.
27. BERBER A. TIDDENS, Wortelrot van *Frimula obconica* veroorzaakt door *Thielaviopsis basicola* (Berk. en Br.) Ferraris. Utrecht 1933.
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30. MARIE S. J. LEDEBOER, Physiologische onderzoekingen over *Cerastomella ulmi* (Schwarz) Buisman. Utrecht 1934.
31. H. G. P. DUYFJES, Het probleem der actieve immunisatie van planten tegen *Pseudomonas tumefaciens*. Utrecht 1935.
32. E. M. VAN ZINDEREN BAKKER, Investigations about the morphology and physiology of *Physalospora cydoniae* Arnaud. Amsterdam 1935.
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34. H. DE JAGER, Ziekteverschijnselen van enkele cultuurgewassen als gevolg van de inwerking van keukenzout. Utrecht 1936.
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36. TH. VAN EEK, Wortelrot van *Viola tricolor* L. Max. hort. Amsterdam 1937.
37. J. G. TEN HOUTEN A. Th. Zn., Kiemplantenziekten van Coniferen. Utrecht 1939.
38. CAROLINE H. KLINKENBERG, Abnormale kurkvorming. Amsterdam 1940.

39. ALIDA JAARVELD, De invloed van verschillende bodemschimmels op de virulentie van *Rhizoctonia solani* Kühn. Amsterdam 1940.
40. K. HARTSUIJKER, Het wetenschappelijk onderzoek van fungiciden. Amsterdam 1940.
41. J. GROSJEAN, Het parasitaire karakter van enige *Polyporaceae*. Amsterdam 1942.

Untersuchungen welche noch in Bearbeitung sind:

Grondontsmetting en grondontsmettingsmiddelen (D. MULDER).

Ziekten van Phlox (J. GUITTART).

De kanker van de wilg (A. MOOY).

Onderzoekingen over bodemschimmels (A. SCHREUDER).

Antagonismen (S. DE BOER).

Wortelrot van vlas (F. HESPE).

Insectenschimmels (P. J. BELS).

(From the Laboratory of Hygiene of the University, Utrecht).

ON THE ACTION OF SULFANILAMIDE

VI. THE ACTION ON BACTERIAL RESPIRATION ¹⁾

by

K. C. WINKLER

(Received January 2, 1942).

In a previous communication we have shown that sulfanilamide, in vitro, acts directly on the cocci (not on the medium) and is not altered before action (5). All suppositions about a transformation of the molecule into an actual germicide (3, 8, 10) or about a blocking of certain food factors of the medium by the drug (7) can thus be dismissed. As sulfanilamide is not directly bactericidal many authors have already supposed an action of the drug on bacterial metabolism, f.i. on some bacterial enzym or ferment. The differences which exist between the metabolism of bacteria in general and their mammalian hosts might then account for the relatively low toxicity of sulfanilamide (12). We have already shown that sulfanilamide does not act on bacterial catalase (4, 9). Trying to obtain evidence about the actual processes of bacterial metabolism sulfanilamide interferes with, we first studied respiration. In an earlier study of the influence of several substances on the action of sulfanilamide in vitro, we had incidentally noted that sulfanilamide is counteracted by glucose (Fig. 1). Thus we did not expect a strong action of sulfanilamide on carbohydrate metabolism.

To obtain further evidence, we studied the action of sulfanilamide on the O₂ consumption of resting bacteria (*Bacterium coli* and *Streptococcus haemolyticus* strain ARONSON) with WARBURG's technique and on the dehydrogenation of a number of hydrogen donators in Thunberg tubes.

Literature.

BARRON and JACOBS (1) reported a distinct action of sulfanilamide on the O₂ consumption. We are of the opinion that the published data

¹⁾ Fifth communication: K. C. WINKLER and H. W. JULIUS, *Antonie van Leeuwenhoek* **7**, 161, 1941.

(7% reduction of O_2 consumption for streptococci, about 10% for some other species and merely for FRIEDLÄNDER's bacillus as much as 23%) do not really permit this conclusion as differences of about

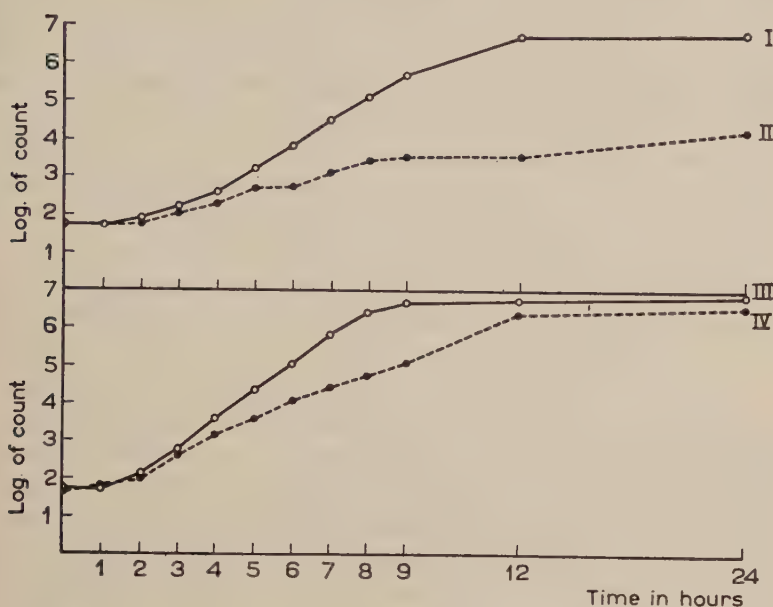


Fig. 1. Influence of glucose (3%) on the action of sulfanilamide (0.1%) on *B. coli* in peptone water.

I = *B. coli* in peptone water

II = idem with 0.1% sulfanilamide

III = *B. coli* in peptone water with 3% glucose

IV = idem with 0.1% sulfanilamide

10% are not very convincing, considering the many difficulties of manometric determinations of respiration especially in the case of streptococci. If the main action of sulfanilamide would consist in an actual blocking of a respiratorial enzym a very clear cut effect could be expected.

Furthermore as sulfanilamide only seems to act on fast growing cultures whilst the action is delayed for some hours, the latter experiments, in which sulfanilamide has been added to resting bacteria and the consumption has been recorded for merely a few hours, can hardly be considered as conclusive.

CHU and HASTINGS (2) for ratdiaphragm and liver, also for gonococcus, *Pneumococcus* type I and II, did not note any significant reduction

of O_2 consumption in the usual concentrations of sulfanilamide. Only in a sulfanilamide concentration of 0.66%, which is near to saturation and about the hundredfold of the concentration active in vitro or possible in vivo, an appreciable reduction of oxygen consumption did occur. For meningococcus a reduction of 17% was observed in more normal concentrations. Such effects can not have a possible bearing on the mechanism of sulfanilamide action.

KEMPNER, WISE and SCHLAGER (6) measured the O_2 consumption of actively growing cultures of *Brucella melitensis* with or without sulfanilamide and related drugs. The O_2 consumption per unit of bacteria was equal in both cases.

No influence of sulfanilamide (0.17%) on the dehydrogenation of glucose was found for pneumococci type I by MELLON and BAMBAS (11).

Materials and Methods.

16 hours old cultures of *B. coli* on plain broth or of *Streptococcus haemolyticus* on 10% ascites infusion broth were centrifuged and the bacteria washed with 0.1 mol phosphate buffer of pH 7.4. The washed bacteria were suspended in buffer, aerated for one hour, and kept in the ice box. Suspensions of streptococci lost their activity very quickly. The suspensions were generally used immediately and in no case after having been kept longer than for 24 hours. For *B. coli*, however, the regression is very much slower and it would have been quite possible to test coli suspensions after a longer period.

O_2 consumption was measured in a differential manometer against a control vessel containing either water or suspension without substrate. The volume of consumed O_2 could be read directly as the volume of mercury necessary to reduce the manometer (filled with BRODIE's solution) to level. The vessels were kept in a water bath of 40° C., and shaken 80 t.p. min.

Dehydrogenation rates were measured in a water bath at 40° C. in Thunberg tubes, containing 1 ml suspension, 1 ml 1% substrate, 1 ml methylene blue 1:5000 and 3 ml phosphate buffer pH 7.4 and 1 ml sulfanilamide solution or 1 ml distilled water.

Results.

I. O_2 CONSUMPTION BY RESTING COLI BACTERIA WITH AND WITHOUT SULFANILAMIDE.

The vessel of each manometer contained 1 ml 4% glucose and 1 ml bacterial suspension with or without sulfanilamide. The control vessels

contained the same suspension but no glucose and in later experiments distilled water as the well aerated suspension did not show any consumption of O_2 in the absence of substrate.

In the described experiment the original coli suspension was diluted with an equal part of buffer solution for manometer I and with sulfanilamide 0.2% in buffer solution for manometer II. The manometers III and IV differed from I and II merely by a dilution of the original suspension 1 : 4. The number of viable bacteria in the four suspensions was determined as:

- I. 273×10^7 per ml (control)
- II. 294×10^7 per ml (with sulfanilamide)
- III. 66.5×10^7 per ml (control)
- IV. 70×10^7 per ml (with sulfanilamide)

which means that I and II as well as III and IV contained bacteria in fairly equal numbers.

O_2 consumption was recorded during 6 hours. The results are given in Fig. 2.

The experiment is one selected out of many which produced similar results. The O_2 consumption by resting coli bacteria is not altered by the presence of sulfanilamide.

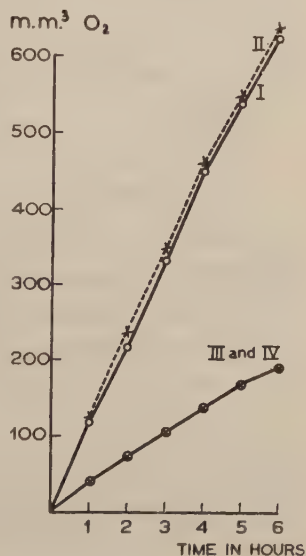
Sulfanilamide shows no bactericidal action on resting bacteria; the bacteriostatic action of sulfanilamide is only distinct on (fast) growing bacteria. Thus it will be necessary to measure the O_2 consumption of resting bacteria grown in sulfanilamide as compared with those prepared from normal cultures.

II. O_2 CONSUMPTION BY RESTING COLI BACTERIA GROWN IN SULFANILAMIDE.

B. coli was cultivated in 1% glucose broth with or without 0.1% sulfanilamide.

Fig. 2. O_2 consumption of resting coli bacteria.

- I = concentrated suspension
- II = idem with 0.1% sulfanilamide
- III = diluted suspension (1 : 4)
- IV = idem with 0.1% sulfanilamide
- 0 = controls x = sulfanilamide



Resting bacteria were prepared as before. The bacteria grown in sulfanilamide were washed with buffer containing 0.1% sulfanilamide and finally suspended in the same medium. For the obtaining of suspensions of equal turbidity it is evident that more broth cultures were needed when sulfanilamide had been present.

The suspensions were diluted as in the preceding experiment, either with equal parts of buffer solution or with buffer solution with 0.1% sulfanilamide for manometer I and II, manometers III and IV containing a final dilution of 1:8.

The numbers of viable bacteria were:

- I. 184×10^7 per ml (control)
- II. 171×10^7 per ml (with sulfanilamide)
- III. 59×10^7 per ml (control)
- IV. 44×10^7 per ml (with sulfanilamide)

The O_2 consumption during 4 hours is plotted in Fig. 3.

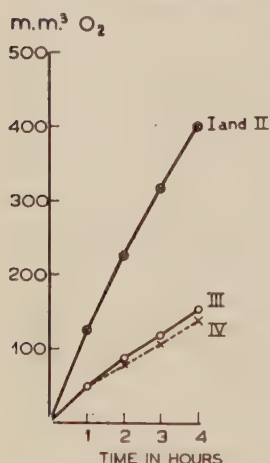


Fig. 3. O_2 consumption of *B. coli* grown in sulfanilamide containing media, compared with normal bacteria.

- I = control, 184×10^7 bacteria per ml suspension
- II = bacteria grown in 0.1% sulfanilamide, 171×10^7 bacteria per ml suspension (with 0.1% sulfanilamide)
- III = control diluted 1:4, 59×10^7 bacteria per ml suspension
- IV = bacteria grown in sulfanilamide diluted 1:4, 44×10^7 bacteria per ml suspension (with 0.1% sulfanilamide)
- 0 = controls x = sulfanilamide

There is no significant difference between sulfanilamide and the controls. The difference in numbers of viable bacteria might explain the very small differences that seem to exist.

Sulfanilamide does not influence O_2 consumption of *B. coli*, even when these were grown in sulfanilamide.

III. O_2 CONSUMPTION OF STREPTOCOCCI.

Suspensions of resting streptococci prepared from cultures in infusion broth with or without glucose did not show any O_2 consumption. O_2

consumption was observed however in cultures on 10% ascites broth or even more so in cultures on tryptic broth. Suspensions were prepared in the same way as for *B. coli* and used the same day. The O_2 consumption by streptococci was much smaller than in the case of *B. coli* and after 24 hours was reduced to still smaller values.

Addition of sulfanilamide to a concentration of 0.02% which is very active as growth curves may learn, did not influence the O_2 consumption of the prepared suspension.

After growing the streptococci for 16 hours in tryptic broth with 0.02% sulfanilamide and suspending the centrifuged cocci in buffer with the same concentration of the drug, there was no difference between the O_2 consumption of the latter suspension and the control suspension, prepared from streptococci cultured without sulfanilamide. As after 16 hours the number of bacteria is much smaller in the culture containing sulfanilamide, much more of this culture was needed to obtain suspensions of equal turbidity.

It was not possible to compare two different concentrations of bacteria as the O_2 consumption was too small and more concentrated suspensions could hardly be prepared from the cultures containing sulfanilamide.

A typical result is shown in Fig. 4. The number of viable bacteria in the sulfanilamide containing suspension was 81×10^6 cocci per ml, whereas the control suspension contained 85×10^6 cocci per ml.

In some instances the streptococci (and also *B. coli*), cultured in sulfanilamide, seemed to consume slightly more oxygen than the control, which might be explained by the fact, that a 16 h. culture of streptococci is already saturated and the bacteria are less active than the cocci grown in sulfanilamidé, which never did reach the saturation point. The same phenomenon was observed sometimes for the rate of dehydrogenation as will be seen below. We conclude that sulfanilamide does not interfere with respiration as measured by O_2 consumption either of *B. coli* or of streptococci.

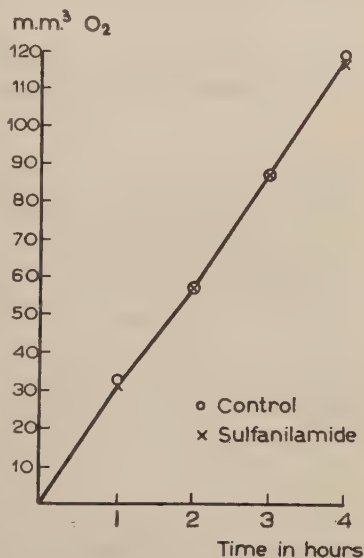


Fig. 4. O_2 consumption of streptococci grown in 0.02% sulfanilamide compared with normal streptococci.

IV. INFLUENCE OF SULFANILAMIDE ON DEHYDROGENATION.

For suspensions of *B. coli*, prepared as before, the periods needed for reduction of methylene blue by means of various hydrogen donators were determined. Controls with (aerated) suspensions without substrate, with sulfanilamide without substrate, with sulfanilamide and bacteria without substrate or with mere substrate, were always negative.

Sulfanilamide showed never any influence on reduction times with the exception of some cases in which the slight enhancing effect already mentioned occurred. Fig. 5 shows the reduction times for different

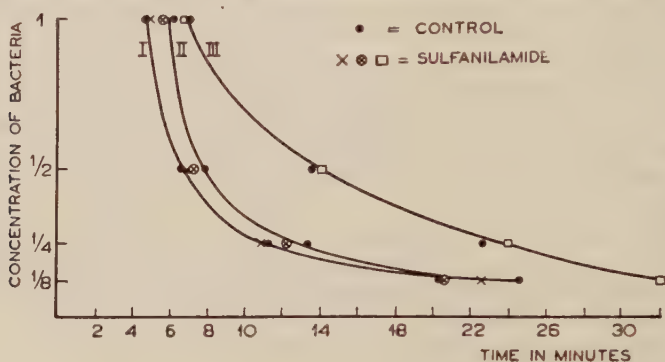


Fig. 5. Reduction time with four dilutions of a suspension of resting coli bacteria, with or without sulfanilamide. I = glucose, II = lactate, III = mannitol.

dilutions of a suspension of *B. coli* with and without sulfanilamide for glucose, mannitol and lactate.

The rates of dehydrogenation measured in suspensions of *B. coli*, cultured in sulfanilamide, were equal to the rates observed for suspensions of normal bacteria. In Fig. 6 reduction

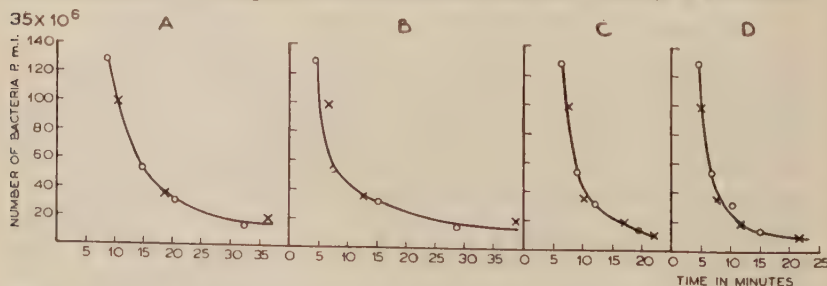


Fig. 6. Reduction time of several concentrations of *B. coli* grown in 0.1% sulfanilamide compared with normal bacteria. A = glycerol, B = mannitol, C = lactate, D = glucose, 0 = controls, x = sulfanilamide.

times are plotted against the number of bacteria; ($\times$) indicating the results obtained with dilutions of a suspension prepared from a culture of *B. coli* containing 0.1% sulfanilamide, (0) indicating results with a suspension of normal bacteria.

Apart from these results with mannitol, glucose, lactate and glycerol, similar results were obtained with formate, pyruvate and succinate.

With streptococci similar results were obtained. No difference was observed on adding sulfanilamide to normal suspensions. Also for streptococci cultured in tryptic broth with 0.02% sulfanilamide no difference in reduction time could be found when compared with normal streptococci. Fig. 7 gives some results with glucose. With maltose, mannitol, lactate and pyruvate similar results were obtained.

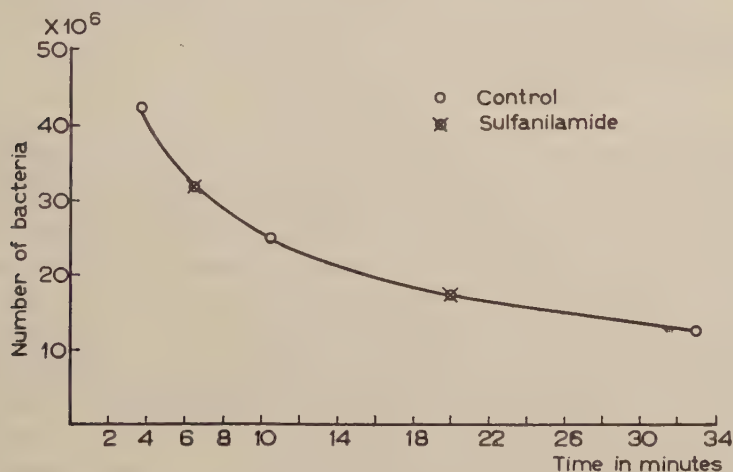


Fig. 7. Reduction time with some concentrations of streptococci grown in 0.02% sulfanilamide compared with normal streptococci.

It is clear that sulfanilamide does not influence the dehydrogenation velocity for the used hydrogen donors. A blocking of bacterial dehydrogenases in general seems improbable.

Discussion.

Sulfanilamide does not reduce the O_2 consumption of the studied organisms for which sulfanilamide, as shown by growth curves, is bacteriostatic, nor does it interfere with bacterial dehydrogenases. It may be inferred that no respiratorial enzymes are inactivated by the drug. When the action of sulfanilamide consists in the blocking of

one or more bacterial enzymes, these have to be looked for in processes of bacterial metabolism other than respiration.

S u m m a r y.

The oxygen consumption of resting *B. coli* or *Streptococcus haemolyticus* (ARONSON) was not influenced by addition of sulfanilamide.

When both organisms were cultured in media containing sulfanilamide and suspended in sulfanilamide containing buffers the oxygen consumption was the same as in control suspensions of normal cultures.

The same conclusions held true with regard to dehydrogenation velocity for various hydrogen donators.

The inference is that sulfanilamide does not interfere with bacterial respiration. The cause of sulfanilamide action must be looked for in some other process of bacterial metabolism.

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(From the Institute for Preventive Medicine, Leiden).

THE CULTIVATION OF FILTERABLE VIRUSES IN VITRO

by

P. H. H. DE BRUYN

1. INTRODUCTION.

The first attempts to cultivate viruses outside the organism were entirely based on the experiences gained in the cultivation of bacteria. These attempts, however, remained fruitless. In contradistinction to the group of bacteria which can be made to live saprophytically and multiply on non-living substrates, for viruses such method could at most induce a conservation during a short period and soon inactivity occurred. Obviously viruses had other requirements which could only be met with after the cultivation of animal cells outside the organism had become possible. Then it appeared that viruses could multiply in vitro, providing living cells occurred in the growth medium. HARRISON (23) succeeded in 1907 in the cultivation in vitro of embryonic frog tissues in lymph. BURROWS (6) substituted in 1910 plasma for the lymph and cultivated embryonic tissues of warmblooded animals. CARREL (7) improved the technique markedly and could arrive at a considerable prolongation of the period during which the tissues could be kept alive by means of periodic supply of fresh culture fluid.

Already in the early stages of the technique of tissue cultures attempts had been made to apply this technique to the investigation of viruses. So LEVADITI (30) succeeded as early as 1913 in keeping up the activity of polyomyelitis virus for monkeys by cultivation outside the body on spinal ganglion in monkey-plasma at 37° C. No multiplication of the virus however was evidenced. STEINHARDT, ISRAELI and LAMBERT (50,51) met in 1913 and 1914 with further success in their experiments with vaccine virus. These investigators cultivated vaccinia virus on small pieces of rabbit's cornea and not only could they state a distinct multiplication of the virus, but they also furnished proof of the indispensability of living cells. If the cornea tissue had been impaired by freezing or by

hypotonic solutions no multiplication of the virus occurred. Although the latter investigations bore on fundamentals of the technique of tissue culture, it was not before 1928—29 that the new technique was applied on a larger scale in virus investigation. Obviously the technique of tissue culture had been too subtle and complicated and it was only after MAITLAND and MAITLAND (34) had presented in 1928 a considerably simplified method, which was still further simplified by LI and RIVERS (31), that tissue culture could occupy the important place in virus investigation it actually does now.

2. THE METHODS FOR THE CULTIVATION OF FILTERABLE VIRUSES.

The methods for the culture of filterable viruses are essentially the same as those used in tissue culture and in fact a virus culture is a tissue culture which has been inoculated with the virus in question.

For a tissue culture a medium is required which contains besides nutritional substances some matter providing support. A mixture of blood plasma and tissue extract will meet both demands. The press-juice of embryos is preferred, as it also contains growth-promoting substances. The plasma is prepared by centrifuging blood, the clotting of which has been prevented either by the supply of an anti-coagulum (heparine) or by paraffination of the glassware. The tissue-extract may be prepared by mincing a chicken embryo of about 8 days, using scissors or a small press constructed for this use. The suspension, prepared in this way, may be diluted with some physiological solution such as Tyrode. The small fragments of tissues are subsequently removed by centrifugation. The supernatant fluid is the tissue-extract.

The plasma clots, whenever a drop of tissue extract is added to a drop in which a tissue fragment freshly taken from the living organism has been suspended. The fibrinal structure of this clot forms the supporting substance and other substances in the plasma and tissue extracts cover nutritional needs. If measures have been taken for the continuous provision of the explant with fresh nutrient fluid and for the elimination of metabolic products (such as washing, subculturing, flowing through) the preparations for a tissue culture are accomplished. All these manipulations have to be carried out under strictly aseptic conditions and any impairment (such as desiccation) of the future explant has to be prevented.

The methods used for virus culture may be divided into three groups:

- a) Cover glass cultures
- b) Cultures in CARREL's tissue-culture flasks
- c) Cultures consisting simply of cell suspensions.

The classification in this sequence partly coincides with the development of virus culture. Merely the former two comply wholly with the already mentioned essentials of tissue culture.

a) **Cover glass cultures.** A drop of plasma is supplied to a sterile cover glass bearing a fragment of tissue in a drop of tissue extract. An inverted hollow vaselined slide is laid down on the cover glass and the slide is turned over. The coagulated culture fluid containing the explant is now hanging on the lower side of the cover glass. This method is essentially the same as the hanging-drop technique used in bacteriology. The cover glass is sealed with paraffine. After one or two days the tissue fragment will be surrounded with a so-called „zône of growth". The renewal of the culture medium and the removal of metabolic products are realized by the „transplanting". With a narrow sharp scalpel a square is cut, containing some of the growth zône. This isolated particle, eventually again divided in two or more particles, can be explanted anew.

It is also possible to start from tissue which has already been infected in the organism and explant the latter. As a matter of course the inoculum has to be bacteriologically sterile.

As a matter of fact this method does not allow of virus production on a large scale. It is due to the necessity of transplantation and probably to the subtlety of the technique as well that this method has not been largely applied in virus investigation.

b) The disadvantages of cover glass culture are for the greater part overcome by the culture in CARREL's tissue-culture flasks. In this case the medium consists of a solid and a liquid phase. The solid phase consists of a coagulum of plasma with tissue (embryo) extract (diluted with Tyrode's solution). The fluid phase is formed by diluted embryo extract. The coagulated plasma covers the bottom of the flask; herein several tissue fragments are imbedded. A thin layer of the fluid phase is supernatant. By periodically supplying fresh fluid the metabolism products can be removed and nutrient substance added. By these means the actual culture is left undisturbed. Next to both these methods the culture may also be inoculated by supplying the virus to the fluid phase. By means of CARREL's flasks appreciably greater amounts of virus may be produced.

c) In contradistinction to the methods of a) and b) the following

methods are wholly adapted to the virus culture and are not suitable for tissue culture. Mere suspensions of tissue are used as substrate; any „solid” medium is lacking.

1) The method of MAITLAND and MAITLAND (34). Here the medium consists of a suspension of minced tissue (chick kidney, rabbit's kidney, rabbit's testis) in a mixture of Tyrode's solution and serum of chicken or rabbit. Originally CARREL's flasks were used but it appeared subsequently that Erlenmeyer flasks of 25 or 50 cc might serve as well. The bottom of the flask is covered with a thin layer of tissue in order to guarantee the oxygenation of the tissue fragments.

2) Even more simple is the method of LI and RIVERS (31). The tissue culture consists here merely of a suspension of embryonic chick tissue (or rabbit's testis) in Tyrode. LI and RIVERS designed a special culture flask, the „collar flask”, in which the back flow of the eventually infected condensation water is caught up by a kind of drain and is thus prevented to contaminate the culture fluid. So contamination of the culture from this origin is prevented. Any physiological solution other than Tyrode's may be used, *viz.*, the solutions of LACKE, DREW and of FRANCIS and MAGILL (14) and even the usual saline. The inoculation may be carried out by adding the virus directly to the cultures.

Studying the problem of the foot-and-mouth disease, FRENKEL and VAN WAVEREN (17, 18) cultivated the causal virus in a completely liquid medium (serum, Tyrode- or RINGER-solution) or in a modified CARREL medium. They could arrive at fairly large quantities of infectious substance by cultivating in ROUX bottles or KOHLE flasks. For thin cultures they started from tissue cultures of embryonic cow- or sheep skin, either kept at body temperature or in the cold during 8 to 14 days. In the latter case good tissue and virus cultures were arrived at. In their passage inoculations they started always from fresh embryonic skin tissues.

All these methods and especially the latter have simplified the technique considerably.

The methods for the cultivation of virus do not wholly agree with those applied in tissue culture. So the methods mentioned under c) do not suit the cultivation of tissue. Apart from the fact that a liquid medium would not allow cytological study, there are many investigators who doubt whether it actually allows of cell proliferation, *viz.*, whether an actual tissue culture is obtained.

Although FRENKEL and JULIUS (15) actually succeeded in the cultivation of vaccine virus in the „flowing-through apparatus” of JULIUS,

this apparatus is too complicated and offers too many opportunities for bacterial infection. That is the reason why it has not been put to practical use.

The partial divergence of tissue culture and virus culture is thus due to the fact that the tissue culture as a substrate for virus culture has been adapted to this special purpose and has developed along its own line.

Whatever method will be applied, there will always exist the need of subculturing on a fresh medium. After an initial multiplication of virus, a decrease will occur in the culture and after a certain period the process will end in complete loss of infective power. This is not primarily due to a dying off of the cells either by exhaustion of the medium or by accumulation of products of metabolism. The multiplying virus itself plays the most prominent part in the impairment of the cells. PLOTZ and EPH-RUSSI (40) have shown that a suspension of embryonic cells remained alive during 12 to 13 days in DREW's solution at 30° C., in the presence of fowl plague virus however merely during 2 days. It appeared that the virus multiplied in the first two days, then a gradual decrease set in and on the 8th day all activity was lost. Before this loss occurs a culture has to be transplanted. The moment when multiplication is optimal is best fitted for this manipulation. The actual moment depends on the kind of virus and varies from 2 to 7 days following inoculation. The virus of the Rous sarcoma is an exception. Cultures of Rous sarcoma will go on producing virus during some weeks. This may be explained by the fact that Rous sarcoma virus, being cultivated in culture of monocytes, does not impair these cells but transforms them into fibroblasts (CARREL and EBELING (8)).

In subculturing one may proceed in various ways. In cover glass cultures the tissue fragment may be divided in some particles, and each of these may be added to a fresh fragment and cultivated further. In the other culture methods a fresh culture may be supplied with some of the original culture fluid. These methods do not allow however of the obtaining of quantitative results. In that case the entire culture has to be minced in a mortar with sand and centrifuged. The virus may be subcultured out of the supernatant fluid. If the titre of the virus suspension used as inoculum of the initial culture is known, next to the dilution of the virus suspension in the culture medium and the amount used as inoculum, the multiplication of the virus can be estimated quantitatively, and any passive transmission of virus in the culture zone may be prevented.

Numerous viruses have been cultivated according to one or other of these methods. The most important work has been carried out after 1930 with culture media consisting either of suspensions of tissue according to MAITLAND and MAITLAND or of embryonic chicken tissue according to LI and RIVERS. The latter forms, as stated by HALLAUER (22) a fairly universal nutrient medium for most kinds of viruses.

3. THE MEDIUM.

The recognition of the fact that viruses can exclusively be cultivated in living cells constitutes for the moment along with their invisibility and filterability one of the most striking features of the viruses.

By this fact the whole group — provided viruses are living agents — is marked as obligatory parasites. We must keep in mind, however, that this is not primarily based on positive results of the cultivation of viruses in the tissue cultures, but that the negative results of the attempts to cultivate on non-living substrata furnish the most substantial evidence. Several investigators have attempted to cultivate virus in cell free medium. EAGLES and MACCLEAN (12) claim to have succeeded. They cultivated vaccine virus in cell free kidney's extract with serum and Tyrode's solution and succeeded in the carrying through of 6 to 7 subcultures in which the virus had multiplied considerably. It is remarkable however that not all the cultures in their experiment presented a multiplication of the virus, but merely a few out of the whole range. Why the virus in those latter cultures multiplied and which conditions play a part in this process remains according to EAGLES (11) still unknown. These experiments have been exactly repeated by RIVERS and WARD (45, 46) and extended over other extracts (rabbit's testis, chick embryo, rabbit's kidney). They could not confirm however the results of EAGLES and co-workers. RIVERS and WARD could affirm, however, that merely small amounts of tissue suffice for a multiplication of virus: 5 to 10 mg per 2 cc fluid and they deem it likely that even smaller quantities might suffice.

Neither could MAITLAND, LAING and LYTH (35) obtain cultures of vaccine virus in a cell free medium consisting of extract of rabbit's kidney.

As for the psittacosis virus MACCALLUM (32) has tried to cultivate it in chick embryo extract with negative results however.

Though numerous other investigators (KRONTOWSKI, JAZIMIRSKA-KRONTOWSKA and SAWITSKA (28), HAAGEN (19), HALLAUER (21)) have attempted to confirm the observations of EAGLES, not one of them could arrive at any positive results.

By still other means it has been attempted to obtain cell free virus cultures: *viz.*, by separating the virus from the tissue culture by a membrane impermeable for the virus (collodion, cellophane) (MUCKENFUSS and RIVERS (37), MUCKENFUSS (36), MACCALLUM (32), LENZ (29)) Neither have any virus cultures been obtained by these means.

Notwithstanding the investigations of EAGLES the opinion is still adhered at generally that the presence of living cells is indispensable for multiplication of viruses. Initially it has been supposed that even this condition would not suffice and that cell proliferation would be required. The simplicity of the substrata of MAITLAND and MAITLAND and LI and RIVERS caused some doubt however; it was considered impossible that any cell proliferation would occur herein. MAITLAND and MAITLAND themselves were of the opinion that their medium did not allow of an actual tissue culture; they note after an interval of some days a disintegration of tissue fragments and autolysis of the cells, along with a multiplication of the virus. RIVERS, HAAGEN and MUCKENFUSS (44) however stated that tissue fragments which had been taken from the MAITLAND medium even after 5 to 6 days and been explanted in a usual medium with plasma, might still proliferate, *viz.*, that viable cells were still present. KRONTOWSKI, JAZIMIRSKA-KRONTOWSKA and SAWITSKA (28) indicate that in the MAITLAND-medium numerous tissue particles may even grow very well on the glass surface of the vessel (compare with the need mentioned before of supporting matter). HALLAUER (22) as well observed cell proliferation in the MAITLAND-medium. Basing on these data cell proliferation is not yet to be dismissed as an indispensable condition for virus culture.

PLOTZ and EPHRUSSI (41) investigated tissue fragments (chick embryo) suspended in DREW's solution at 37° C. histologically. It appeared that merely in the first two days mitoses occurred. After incubation of such a cell suspension during 5 days at 37° C. fowl plague virus was added. After such an interval (until the 12th or 13th day see above) tissue fragments will still contain numerous living elements. After two days similarly treated cultures were again inoculated. In this way 10 passages were made; the titre amounted to 1:1.000.000. It is however questionable whether this multiplication of virus had exclusively occurred in the presence of merely surviving cells. In tissue culture namely amitotic cell cleavages may occur frequently, and an absolute increase of cell number may not be precluded.

HECKE (24, 25, 26) cultivated the virus of foot-and-mouth disease in cover glass cultures on the skin of guinea-pigs at 37°, 30° and 26° C. At

37° C. tissue culture as well as multiplication of virus occurred. At 30° C. increase of tissue could only rarely be noted and even then a very slight one; the multiplication of virus however had only decreased in small measure, whilst at 26° C. both processes were at a standstill. Although on this base it might be accepted that cell proliferation is not indispensable for the multiplication of virus, other publications however indicate such an influence. So it has been noted by HALLAUER (21) that the multiplication of fowl plague virus occurs much more slowly in merely surviving tissue (MAITLAND culture) than in actually proliferating CARREL cultures; the resulting concentration of virus however was the same in both cases. PLOTZ (39) however noted that a same virus was 10 to 100 times more active in proliferation cultures than in cultures consisting of merely surviving cells.

The presence of living cells is not the only condition for a virus culture. The nature of these cells is also a factor of major importance. In view of this DOERR (10) has divided the viruses in two groups:

- a) Viruses which need *in vitro* cells of the same animal species they can infect *in vivo*.
- b) Viruses which *in vitro* are not restricted to cells of some particular animal species.

More recent investigations of HALLAUER (22) have proved however that such a classification cannot be founded merely on culture experiments. He has shown that the virus of fowl plague, which when freshly taken can solely be cultivated in cells of its natural host (chicken, duck, goose, pigeon) and according to this should be placed in group a), may however be cultivated in embryonic mammal-tissue (mouse) provided that some passages in embryonic tissue have preceded.

So this problem is apparently less simple than it was deemed originally and the more so as the type of cell plays also an important part. HALLAUER cultivated the fowl plague virus in pure cultures of cell-types, monocytes, fibroblasts and osteoblasts. He could observe multiplication of virus in none but epithelial cells.

HECKE (24, 25, 26) as well as FRENKEL and VAN WAVEREN (17, 18) have shown that the foot-and-mouth disease virus can only multiply in epithelial cells, preferably in skin epithelium. These scientists noted that the latter virus did not multiply in embryonic chick tissue.

The virus of the Rous sarcoma can be cultivated solely in cultures of monocytes of the hen (CARREL (7)). Up till the present the psittacosis virus can be cultivated only in fibroblast cultures (BEDSON and BLAND(4); BLAND and CANTI (5)). All these data help to explain why chick embryo

is such a favorable substrate for the cultivation of viruses. Moreover it is an important factor that this matter can be so easily sterilized. It is curious that on embryonic chick tissue viruses may be cultivated which lack in vivo affinity for this animal species, e.g. vaccine virus, influenza virus, yellow fever virus etc. For the present virus- and tissue medium appear to be merely slightly connected. Only by means of systematic experiments deeper insight might be gained.

4. THE VIRUS.

So it is evident that the multiplication of virus depends in a large measure on the substrate. In recent investigations (THEILER (54, 55), SMITH and THEILER (49), HALLAUER (22)) it has been demonstrated that the virus may be connected with the medium in still another respect, *viz.*, as far as its variability is concerned. It appeared possible to influence the properties of the virus in vitro. This is shown very clearly in the culture experiments of THEILER (54 or 55) with the yellow fever virus. THEILER cultivated the pantropic (viscerotropic and neurotropic) yellow fever virus in various tissues, namely in embryonic mice tissue, testis tissue and embryonic tissue of chicken, the latter after brains and spinal cord had been removed. The pathogenic properties of the cultures were tested continuously by extra- and intravenous inoculation of monkeys and intracerebral inoculation of mice. The results were that the pantropic virus, when cultivated on embryonic mice tissue preserved its neurotropic properties, showing however a decrease in viscerotropy. The cultivation on testis tissue did not cause a decrease in neurotropy for monkeys, for mice however it did so slightly; the viscerotropic properties however had decreased markedly. When however the culture medium contained embryonic chick tissue, the nervous system of which had been removed, the viscerotropic properties decreased considerably along with the neurotropic ones. So the variability of the yellow fever virus, which had already been recognized in vivo, is apparently a feature which stands out clearly in the tissue culture as well.

The question may now be raised why it is that the pantropic yellow fever virus loses thus its neurotropy in embryonic tissue after removal of the nervous system. There will be the tendency to ascribe this to the absence of nervous tissue. When it is taken into account however that the results of cultivation in testis tissue do not wholly agree with those of cultivation in chick tissue without nervous tissue, such a conclusion may seem premature. It has to be considered whether the chick tissue as such might be the actual cause. The fact, however, that the presence

or absence of nervous tissue might play an important part, is substantiated by the experiments of SMITH and THEILER (49) who cultivated strains of yellow fever on embryonic mice brains, which resulted in a distinct increase of the neurotropy. Similar changes in the pantropic virus can be induced *in vivo* by means of intracerebral passages in mice (THEILER (53)).

Also for fowl plague virus HALLAUER (22) noted a variability induced by the medium. Here again the occurrence of nervous tissue in the medium and the neurotropy appeared to coincide. It will be clear now that tissue culture which offers so many possibilities as to variation in the substrate opens wide perspectives for experiments of such nature.

When the indispensability of living cells for the multiplication of virus is once taken for granted, the question may be raised of what nature the function of these cells may be. This question touches upon the much disputed problem of the nature of viruses themselves. The tissue culture offers a new possibility to go deeper into the relation between living cells and virus. As the first thing it could be determined in which part of the culture the virus multiplied. The experiments in which the tissue was kept separated from the virus by a membrane impervious for viruses, had indicated already that no action at distance is the cause, but that a direct contact is indispensable. Experiments in which the virus content of cells and culture fluid was estimated separately show a similar trend. The results of these experiments (MACCALLUM (32), HECKE (26), SMITH (48)) indicate that the cells although perhaps not solely containing the virus, are surely richest in content.

It is harder to make out whether adsorption of virus or an actual intracellular multiplication of virus takes place here. The fact, that immune serum will not influence the multiplication of virus when the latter has been supplied to the culture previous to the immune serum, is claimed as a protection against the immune serum by means of the intracellular localisation of the virus (ANDREWES (1, 2), MAGILL and FRANCIS (33), ROUS, MACMASTER and HUDICK (47)). It is suggested then that the virus must have had the opportunity to penetrate into the cell (actively or passively?), before the immune serum might display its neutralizing action. Although further investigations are needed to settle how far this conclusion may be justified, it has to be admitted that for the present this interpretation is the most tempting.

A surer base is gained for some kinds of virus, in which the intracellular localisation of elementar bodies in the tissue cells can be demonstrated microscopically (psittacosis virus, vaccine virus, rickettsia).

The possibility of cultivating virus apart from the organism has led to attempts to gather further data about metabolic phenomena eventually induced by viruses. Not any influence however of the virus on the metabolism of the cell could be reported — except in case of actual degeneration — nor any metabolic activity of the virus itself (PARKER and SMYTHE (38)). It has been stated, however, and this might be expected in view of the indispensability of living cells, that multiplication of virus is bound up with the respiration of the cell. MAITLAND, LAING and LYTH (35) have reported the remarkable fact that a correlation exists between metabolic activities of various kinds of tissue and the multiplication of virus or between type of cell and multiplication of virus.

So it is made clear that the cultivation of viruses in vitro is otherwise significant for theoretical research than e.g. the cultivation of bacteria. The tissue culture offers the possibility to approach in some measure natural conditions and to gain in this way some idea of properties and nature of viruses. We merely mention the study of immunology (STOEL (52)). Moreover it covers in its practical application another field than does the cultivation of bacteria.

As early as 1927 CARREL and RIVERS (9) indicated that tissue culture might be an easy and inexpensive method for the obtaining of a highly concentrated and sterile substance for vaccination against smallpox. Since then this has been repeatedly realized (RIVERS (43), HERZBERG (27), PLOTZ and MARTIN (42)) with good success. FRANCIS and MAGILL (13) as well have applied tissue culture for the gaining of substance for immunisation experiments with influenza virus. For a wider application however the latter advantages could not counterbalance the technical difficulties of virus culture.

Virus culture may be of great practical value for the obtaining of modifications of virus suitable for vaccination.

In this connection we mention once more the investigation of THEILER (54, 55). By means of cultivation of the pantropic yellow fever virus in embryonic chick tissue without neural tissue he succeeded in the obtaining of a strain, the pathological properties of which were much weakened whilst the antigenic properties were left quite undisturbed. For immunizing use the strain will be of greater value than the neurotropic mice strain, which, although lacking the viscerotropy of the pantropic strain, is potentially more dangerous for man by its increased neurotropic affinity.

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ON BACTERIA DECOMPOSING CHOLESTEROL

by

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1. INTRODUCTION.

The general occurrence of cholesterol in animal cells and secreta leaves scarcely any doubt as to its paramount physiological significance. Nevertheless, our knowledge regarding its function in animal metabolism is still very restricted. It may only be deemed certain that there are animal cells which are able to synthesize and also cells which are able to decompose this compound.

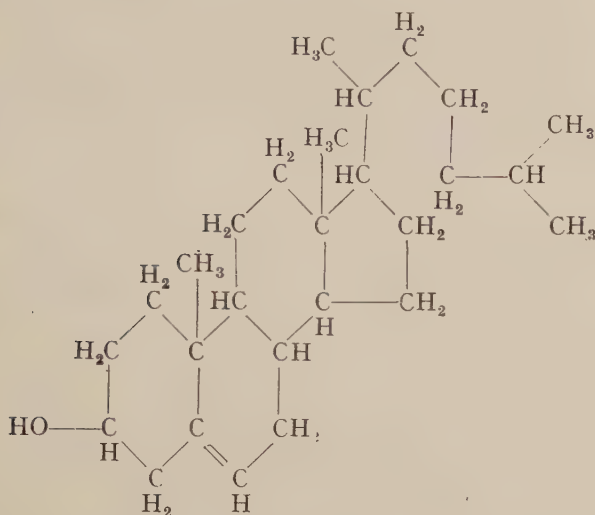
The practical insolubility of cholesterol in water renders the obtaining of an insight into the mechanism of this breakdown process very difficult. In this respect cholesterol cannot be rightly compared with the fats, since a simple hydrolysis is able to convert these latter compounds into more or less water soluble components. To the contrary the breakdown of cholesterol will ask for a direct action of the desmolytic catalysts of the cells, or in other words this compound must as such be subject to reactions of an oxido-reduction type.

Now it is a general experience that the diversity in the metabolic activity of the microbe world is so pronounced that it is nearly always possible to find microbes which are especially adapted to a definite metabolic process.

For this reason it seemed promising to make a search for micro-organisms active in the decomposition of cholesterol. The consideration that the general process of mineralisation must necessarily include the decomposition of a compound like cholesterol, so widely occurring in nature, puts the actual existence of such organisms quite out of doubt. Moreover, there are a few places in literature where, more or less incidentally, reference is made to an actually observed decomposition of cholesterol by bacteria.

It is not surprising that investigators studying the decomposition

of paraffins by bacteria have been tempted to answer the question whether their cultures also had the ability to attack the cyclic hydrocarbon skeleton of the cholesterol molecule:



In 1913 this question was for the first time answered in the affirmative by SÖHNGEN (5) who in his fundamental study on the bacterial decomposition of paraffins incidentally reports that the various *Mycobacterium* species isolated by him from enrichment cultures with paraffin were also able to grow in a medium with cholesterol as a sole source of carbon.

In an elaborate study on the properties of the bacteria of the genus *Mycobacterium* HAAG (1) also remarks that they all show an, albeit scanty, growth in a medium in which cholesterol was the sole organic component. Elsewhere, however, the same author (2) stated more explicitly that both *Mycobacterium testudo* and *Mycobacterium lacticola* grew satisfactorily in such a medium.

We must conclude, however, that until now the evidence for the decomposition of cholesterol by bacteria is only of an indirect nature *viz.*, exclusively based on growth observations. This criterion has to be deemed as rather dangerous, since a slight amount of impurities present either in the cholesterol preparation itself, or in some other component of the medium may well have been responsible for a rather considerable proliferation of the bacteria in question. For it is well known to all workers in this field that the nutritive demands of the saprophytic mycobacteria are quite easily satisfied.

For this reason it seemed worthwhile to reinvestigate the problem of bacterial decomposition of cholesterol, and to attempt to bring more direct proof for its disappearance under the influence of bacterial life. It has also to be taken in consideration that until now the bacteria which were tested as to their ability to decompose cholesterol had not been especially isolated for this purpose. It seemed probable that enrichment cultures in a cholesterol medium might lead to the isolation of species more pronouncedly adapted to the accomplishment of the task of cholesterol decomposition.

2. ENRICHMENT EXPERIMENTS IN MEDIA CONTAINING CHOLESTEROL AS A SOLE SOURCE OF CARBON.

In order to obtain a good dispersion of the cholesterol in the medium I proceeded as follows.

A solution of 1.25 g cholesterol in 60 ml acetone was slowly poured into 250 ml of a hot solution of 0.1% $(\text{NH}_4)_2\text{SO}_4$ and 0.1% KH_2PO_4 in tapwater. The liquid was then boiled until the last trace of acetone had disappeared. The pH value of the medium was 5.9.

The cholesterol in the medium had finally precipitated in a flocculent state.

Erlenmeyer flasks measuring 200 ml were each provided with 50 ml of this medium and inoculated with garden soil.

Various incubation temperatures, *viz.*, 30°, 37°, 45° and 55° C., were applied. After 1 to 2 weeks a visible growth occurred in the greater part of the flasks; if so 1 ml of the contents of each flask was transferred into a second flask with the same, sterilized medium.

In these secondary flasks too a certain amount of bacterial growth was observed in all cases, but it remained doubtful whether this had to be ascribed to a decomposition of the cholesterol.

A markedly different behaviour was, however, observed in one of the media which, in contrast to all others, had been subjected to a continuous shaking during its incubation at 30° C. Here the amount of solid cholesterol seemed to diminish in the course of the incubation, while a luxurious bacterial growth appeared. Transfers of this enrichment culture into fresh media which were as well shaken showed the same feature.

In these cultures numerous bacteria could be detected microscopically, most of which proved to be immotile rods. As it was not always easy to distinguish between bacteria and the small cholesterol particles of the suspensions it was found helpful to make stained preparations.

Before fixing and staining the smears were freed from cholesterol by dipping the air-dried preparations for one minute in chloroform.

In order to get an idea regarding the influence of the additional nutrients upon the bacterial development inoculations were made from one of the transfers into flasks with different cholesterol media.

The first medium consisted of a stable suspension of cholesterol in water to which solely 0.5% of yeast autolysate had been added. This cholesterol suspension was prepared according to prescriptions given by PORGES and NEUBAUER (3) and by REMESOW (4).

The second medium was as the former, but now 0.2% $(\text{NH}_4)_2\text{SO}_4$ and 0.1% K_2HPO_4 were added. As a result of this addition the suspended cholesterol flocculated, giving rise to a fine precipitate.

A third flask contained a similarly prepared suspension of cholesterol with 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.1% K_2HPO_4 and traces of MgSO_4 , CaCl_2 and $\text{Fe}_2(\text{SO}_4)_3$. This time the suspension did not flocculate after the addition of the salts.

In all cases bacteria developed well and quickly. Meanwhile the various media seemed to have favoured the development of different kinds of bacteria present in the original enrichment culture. The colour of the first flask turned pink, the second liquid remained colourless and the third adopted a greenish colour. I therefore made transfers of the three cultures into separate flasks, thus obtaining three series of crude cultures which, nevertheless, originated from one and the same enrichment culture.

In the further work it was found that the degree of dispersion of the cholesterol in the medium was of little importance with regard to the rate of growth. It soon appeared, that the stable cholesterol suspension, representing the finest possible dispersion, and the media with the cholesterol precipitate sustained growth equally well. It was, therefore, tempting to investigate whether it was possible to avoid the rather cumbersome operations involved in the preparation of the stable suspensions and the finely divided precipitates.

As a matter of fact quite satisfactory bacterial development proved to take place in a medium containing solid cholesterol — previously powdered in a mortar — and having the following composition:

cholesterol	3 g
$(\text{NH}_4)_2\text{SO}_4$	2 g
K_2HPO_4	1 g
MgCl_2	0.2 g
tapwater	1 litre
(pH adjusted to 7.2)	

This medium was sterilized by autoclaving at 120° C. for 15 minutes.

After a shaking experiment the cholesterol in blank cultures partly gathered at the surface of the clear liquid, forming a white ring along the wall of the vessel. Cultures in which the cholesterol had been attacked were of quite another aspect: here the remaining cholesterol — usually only the coarser particles — had precipitated.

In some experiments a small quantity of yeast autolysate was added to the medium in order to provide for growth substances, but no favourable effect of this addition could be ascertained.

It seemed of importance now to bring definite proof for the consumption of cholesterol in the crude cultures.

To this aim 20 ml of a homogenous cholesterol suspension prepared as indicated before were brought in 10 Erlenmeyer flasks measuring 100 ml. Then the necessary mineral salts and also 0.1 ml yeast autolysate were added to each flask. Sterilisation entailed a flocculation of the cholesterol. Six of the flasks were inoculated, using each time one loopful of the three different series of enrichment cultures mentioned above. Four flasks remained sterile and acted as controls.

After 28 days of incubation at 30° C. under continuous shaking all inoculated flasks showed a profuse growth, whilst the cholesterol precipitate had clearly diminished.

Now the contents of all flasks were exhaustively extracted with ether. The extracts of each flask were collected in a weighed beaker, and the ether then evaporated.

The ether-soluble residue having thus been determined, the cholesterol was gravimetrically estimated with the aid of digitonine in the following way:

The whole residue or at most 20 mg of it was dissolved in 14 ml of aethanol 96%. On a waterbath this solution was heated to a temperature slightly below the boiling-point. Then a boiling solution of 100 mg digitonine in 12.5 ml aethanol of 96% and 1.75 ml water was added while the mixture was stirred with a glass-rod. On cooling the digitonide crystallized gradually. The beakers were kept covered with a watch-glass for one night and then the precipitate was filtered in a dried and weighed glass-filter (Jena G 3). The precipitate was first washed twice with 2.5 ml of CAMINADE's liquid i.e. a mixture consisting of 73% acetone, 18% water and 9% aethanol (96%), and afterwards twice with 12.5 ml ether. Finally the filter was dried in an exsiccator in a vacuum of about 15 mm Hg above calcium chloride and paraffin till constant weight.

The quantity of digitonisable sterol is about $\frac{1}{4}$ of the quantity of found digitonide, but the factor depends to a certain extent on the quality of the used digitonine. To find the correct value some analyses were carried out with known quantities of cholesterol between 10 and 20 mg.

Thus a standard-curve was produced from which the quantity of cholesterol in the weighed digitonide-precipitates of the subsequent analyses could be derived directly. With the aid hereof the total amount of cholesterol present in the original residue could easily be ascertained.

The results are collected in the following Table.

Utilisation of cholesterol in transfers of enrichment cultures.
Incubation at 30° C. during 28 days.

A. Inoculated flasks			B. Control flasks	
residue mg	cholesterol mg	cholesterol %	residue mg	cholesterol mg
14.1	3.9	9	45.5	42.5
5.6	0.0	0	44.4	42.2
16.0	1.9	4	48.8	42.6
17.5	2.7	7	44.3	41.5
11.9	1.5	3		average
27.8*	5.6*	13*		42.2

* The incubation period of this flask was only 13 days.

The figures in the Table do not need further comment. They prove that in all inoculated flasks incubated for 4 weeks nearly all the cholesterol present had been consumed by the bacteria. Even in the culture which had only be incubated for 13 days by far the greater part of the cholesterol added had disappeared.

3. THE ISOLATION OF THE BACTERIA RESPONSIBLE FOR THE CHOLESTEROL CONSUMPTION IN THE ENRICHMENT CULTURES.

Fresh transfers of the various enrichment cultures were made in the medium with solid cholesterol previously mentioned.

During ten days bacterial development was carefully followed both by direct microscopical examination and by the preparation and examination of stained smears. At regular intervals streak cultures were made on plates of peptone agar with 1% glucose which were incubated at 30° C. Several types of colonies developed on these plates. Pure cultures were made in the usual way from those colonies which

on microscopical examination corresponded morphologically with the bacteria found to prevail in the enrichment culture at the moment that the streaks had been made.

The pure cultures obtained in this way were directly tested as to their ability to grow in the cholesterol medium and to bring about the characteristic visual change in the appearance of this medium.

Among several cultures isolated two strains stood out by a pronounced consumption of cholesterol (strains I and II). Later on I succeeded in the isolation of a third strain (strain III) which in some respects differed markedly from the two previously isolated ones. Although this strain developed well in the cholesterol medium and proved a powerful decomposer of cholesterol, its growth on the peptone sugar medium was merely very poor. Gradually, however, the organism adapted itself somewhat to this medium, although the rate of development remained slow.

A further examination of the three strains showed that all of them belong to the genus *Mycobacterium* Lehmann et Neumann¹⁾.

It was found that they were all obligately aerobic, non-motile rods. Endospore formation was never observed; gelatin was not liquefied. Moreover, they proved to be GRAM-positive and acid fast (10 seconds in 5% sulphuric acid).

Strain I and II showed both straight to slightly curved rods, $1\ \mu$ in diameter and varying in length from 4 to $8\ \mu$. In the older cultures in liquid media coccoid bodies, about $1.5\ \mu$ in diameter, prevailed.

Strain I showed great resemblance to the strain of *Mycobacterium lacticola* present in the laboratory collection; in peptone water with 1% glucose an abundant sediment was formed, whilst pellicle formation was only slight. A characteristic feature of this strain was the production of a brownish pigment which contrary to the usual behaviour of pigments formed by mycobacteria diffused into the medium. Apart from this property the resemblance between strain I and *Mycobacterium lacticola* was such that I feel justified to consider the organism as a chromogenous variety of the former species.

Strain II proved to have so many properties in common with the RABINOWITSCH-strain of *Mycobacterium berolinense* that there was all reason to consider the strain to be identical with this species. In peptone water with 1% glucose a characteristically wrinkled pellicle was formed

¹⁾ In carrying through the identification of the strains I have much profited from the expert assistance of Miss A. L. SCHULING to whom I wish to express here my most sincere thanks.

next to a slight sediment, the rest of the medium remaining clear.

Strain III appeared in the cholesterol medium in all phases of its development as a slender rod, sometimes slightly curved at the ends and measuring 0.2×1.5 to 2μ .

In broth a pellicle is formed together with a sediment, the liquid between remaining clear.

On peptone glucose agar growth is slow, but finally the streak becomes thick, raised and gradually spreading, presenting at last a characteristically wrinkled to nodular, greasy and glistening surface. If exposed to daylight the centre of the streak shows a pink coloration.

Since the general aspect of the cultures differs from that of all other *Mycobacterium* species present in the collection of the „Laboratorium voor Microbiologie" at Delft, and taking moreover into consideration the cultural properties described above, there is reason to claim the organism to be a new species. The name of *Mycobacterium cholesterolicum* seems suitable in a view of its strongly developed activity towards cholesterol.

The fact is undoubtedly worth stressing that the isolation of active cholesterol decomposers out of the enrichment cultures has led in all cases to species of the genus *Mycobacterium*. For it is exactly for representatives of this genus that both SÖHNGEN and HAAG have claimed the property of cholesterol decomposition. Nevertheless, it would be premature to conclude that this property is restricted to *Mycobacterium* species. In this connection we remind of the fact that SÖHNGEN has found on the one hand that many of these species are able to attack paraffins, but on the other hand has shown that enrichment cultures at low temperatures led to the development of *Pseudomonas* species having the same property.

Another question is whether all *Mycobacterium* species are able to decompose cholesterol. I am unable to answer this question definitely, but a preliminary experiment showed that the strains of *Mycobacterium phlei*, *Mycobacterium lacticola*, *Mycobacterium berolinense*, *Mycobacterium salmonicolor* and *Mycobacterium rubrum* present in the laboratory collection all developed quite satisfactorily in shaking cultures in the mineral cholesterol medium.

If it would turn out that the ability to decompose cholesterol is a property common to all *Mycobacterium* species this property may become useful for the demarcation between the representatives of the genera *Mycobacterium* and *Corynebacterium*.

Summary.

It has been definitely proved that in suitable enrichment media in which cholesterol is the only source of carbon this compound disappears with relatively great speed. Continuous shaking of these cultures greatly favours their development. From the enrichment cultures three *Mycobacterium* species have been isolated which proved to be able to attack this compound also in pure culture. One of these species which possesses this property in a very marked degree does not seem to have been described earlier. For this species the name of *Mycobacterium cholesterolicum* has been proposed.

Strains of *Mycobacterium phlei*, *M. lacticola*, *M. berolinense*, *M. salmonicolor* and of *M. rubrum*, taken from the laboratory collection, were also able to decompose cholesterol.

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ARE THE TOXIC PROPERTIES OF A STAPHYLOCOCCAL FILTRATE MANIFESTATIONS OF ONE OR MORE CONSTITUENTS?

by

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(Received March 2, 1942).

1. INTRODUCTION.

The phlebolethal, dermonecrotic and haemolytic properties of a staphylococcal filtrate are believed by some to be caused by one substance, although there is by no means a consensus of opinion on this point.

BURNET (2), who was the first to show that the addition of formalin to a staphylococcal toxic filtrate causes its detoxification without damaging its antigenic property, favours the view that the above-mentioned activities are all due to the same constituent. This opinion is based principally upon his observation that antitoxic sera, prepared in different ways, show a constant relationship between their anti-haemolytic, antilethal and antinecrotic activities. In detoxification experiments BURNET and FREEMAN (3) found „that the skin toxic effect falls *pari passu* with the loss of haemolytic power”. GROSS (6) also believes that the toxic properties of staphylococcal filtrates are caused by one substance. GENGOU (4) and PARISH and CLARK (9) are of the same opinion. BURKY (1) suggests that the lethal factor and haemolysin are independent of each other: the former is much more stable than the latter. Loss of haemolytic activity is sometimes associated with an increase in toxicity. PARKER (10, 11) on the basis of adsorption experiments with erythrocytic stroma, concludes that the necrotoxic action is caused by a constituent in the filtrate, which is different from the haemolytic.

NEISSER and WECHSBERG (7) had observed that, at a temperature of 0° C., the haemolysin is fixed by the erythrocytes, whereas haemolysis does not take place. By carrying out the experiment at this temperature

and by examining the supernate, TIMMERMAN (13) was able to demonstrate that the haemolytic and the dermonecrotic potencies of the staphylococcal toxin had both decreased considerably. This indicates that the erythrocytes adsorb the haemolytic and the necrotic substances. It does not appear justified however, to conclude from this observation that both activities are caused by the same substance.

The object of the present experiments was to clear up, if possible, this disparity in the results.

Different quantities of formalin were added to a certain staphylococcal toxic filtrate and the mixtures incubated at various temperatures. At regular intervals the haemolytic, dermonecrotic and phlebotlethal activities were measured. This procedure permitted to answer the question whether the three toxic factors disappeared at the same rate, or whether different courses were followed. If the latter were found to occur, it would point strongly to different constituents as causing the different activities.

2. METHODS AND MATERIALS.

Strain. The toxic filtrate was produced from a wood-46-strain. This particular strain was used, because it produces very little or no β -toxin (GLENNY and STEVENS (5)).

Production of toxin and its titration. Toxic filtrates were prepared by growing the staphylococcus in an atmosphere of air, containing 20 percent of carbon dioxide, on the medium recommended by PARISH and CLARK (8, 9) and modified slightly by TIMMERMAN (13). The cultures were incubated at 37° C., and filtered through Seitz filters. The pH of the filtrate was adjusted to 6.8, if necessary.

One special batch of toxin, prepared in the manner described above, was used throughout the experiments. It possessed the following properties. No β -toxin was present. It had no haemolytic action on human chromocytes (ROY (12)). The smallest dose which, after intravenous injection, constantly killed mice, weighing from 15 to 18 g, within 24 hours was 0.01 cc (M.L.D.).

The haemolytic activity on rabbit cells was titrated as follows. A 2 percent solution of washed rabbit chromocytes in physiological salt solution was used. The toxin was also diluted in physiological saline. Each tube contained 1 cc of toxin dilution and 1 cc of diluted washed cells. Readings were made after one hour at 37° C., followed by one hour at room temperature. The last tube which showed complete haemolysis was taken as endpoint. Thus, the minimal haemolytic dose (M.H.D.)

was defined as the smallest quantity of toxin which would produce complete haemolysis of the above-mentioned amount of rabbit cells. Throughout the experiment cells from the same rabbit were used (TIMMERMAN (14)). The M.H.D. of this particular toxin was 0.0008 cc.

In order to determine the dermonecrotic properties, 0.1 cc of suitably diluted toxin was injected intradermally into guineapigs. White or cream animals, weighing from 350 to 400 g, were chosen. Readings were made after 24 and 48 hours. The minimal necrotic dose (M.N.D.) was defined as the smallest quantity of toxin which produced definite necrosis (0.5×0.5 cm) within 48 hours. The largest quantity of toxin, which was injected, amounted to 0.100 cc. The M.N.D. was 0.002 cc.

3. EXPERIMENTAL.

The influence of incubation at 37°C ., without the addition of formalin, on the three toxic properties is presented in fig. 1.

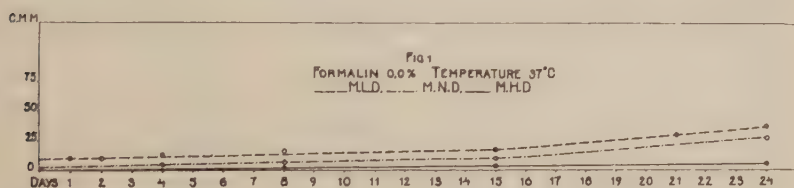


Fig. 1.

These curves show that, in 24 days, the M.L.D. rose from 0.01 cc to 0.035 cc, the M.N.D. from 0.002 cc to 0.025 cc, and the M.H.D. from 0.0008 cc to 0.005 cc. Thus, the process of „toxoiding” proceeds slowly, and the curves do not suggest that the losses of the toxic activities run along different lines.

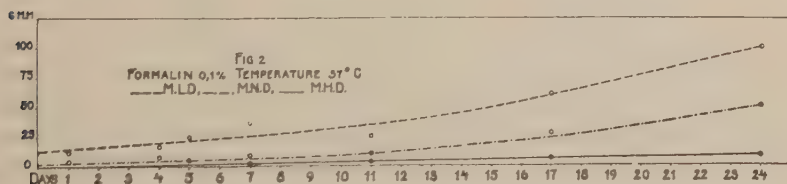


Fig. 2.

Fig. 2 gives the curves when 0.1 percent of formalin had been added to the toxin. Compared to fig. 1, these curves show, that the detoxifying-process ran a quicker course. After 24 days, the M.L.D. amounted to 0.100 cc, the M.N.D. to 0.050 cc, and the M.H.D. to 0.007 cc. Thus,

the M.L.D. increased 10 times, the M.N.D. 25 times, and the M.H.D. 9 times approximately. There were still no appreciable differences in the behaviour of the toxic properties.

When 0.25 percent of formalin was added however, the results were very different, as is shown in fig. 3. After 3 days' incubation at 37° C., the M.L.D. could no longer be determined, since the largest quantity of toxin which was injected into the tail vein (0.500 cc) did not kill mice. The same applied to the M.N.D. for after the same period of time 0.100 cc of toxin did not produce necrosis, although necrosis was produced after merely 2 days incubation.

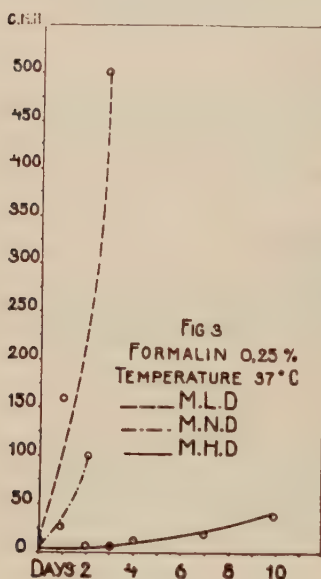


Fig. 3.

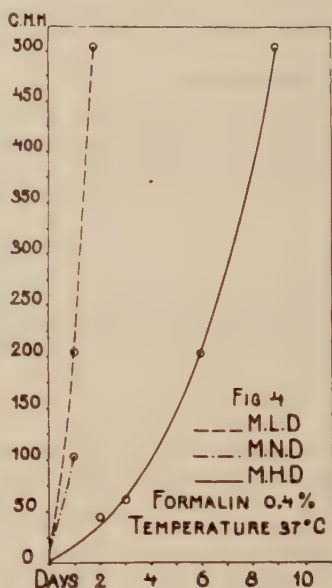


Fig. 4.

The decrease in the haemolytic potency, on the contrary, proceeded much more slowly. After 3 days incubation, the M.H.D. rose from 0.008 cc to 0.005 cc only. Although 0.25 percent of formalin damaged the haemolytic activity more than did 0.1 percent, the influence of increasing the content of formalin was much stronger on the lethal and necrotic properties of the toxin.

Finally, when 0.4 percent of formalin was added, the haemolytic power was also impaired considerably, as shown in fig. 4. The lethal and necrotic power disappeared sooner than when 0.25 percent of formalin had been added. After 1 and 2 days incubation, respectively, the same

decrease was found as after 2 and 3 days, respectively, when 0.25 percent was added. The haemolytic curve is very much steeper, too. After 9 days, the M.N.D. reached 0.500 cc, whereas — as is shown in fig. 3 — with 0.25 percent of formalin the M.H.D. reached 0.035 cc after approximately the same period.

From the experiments described above, it appears that the phlebolethal and the dermonecrotic activities of this toxin behave more or less in the same way. Thus, a steep rise in the „lethal” curve is accompanied by a parallel rise in the „necrotic” curve. On the contrary, the „haemolytic” curve does not always follow the same course. This is evident, especially, when 0.25 percent of formalin has been added.

With a view to obtaining a clearer insight into the process, two more experiments were carried out with the same toxin. For these tests 0.25 percent and 0.4 percent of formalin were added. The mixtures were incubated at 22° C. The results are shown in fig. 5 and 6. In fig 5 ex-

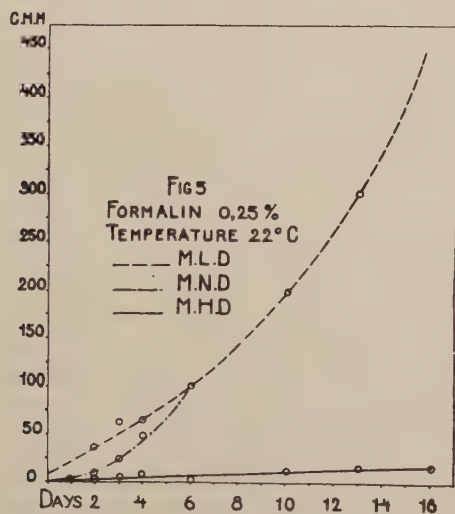


Fig. 5.

periments are recorded involving the same amount of formalin as those shown in fig. 3. This effect must have been caused by the lower temperature of incubation. It appears that the higher temperature had a greater and quicker deleterious influence on the lethal and the necrotic properties than on the haemolytic potency. The differences in the steepness of the first two curves are more marked than in the case of the haemolytic

curves. During the first 4 days of incubation, both haemolytic curves follow practically a same course, whereas the lethal and necrotic curves are much steeper when the temperature of incubation was 37° C. Even when 0.4 percent of formalin had been added (fig. 6), the haemolytic

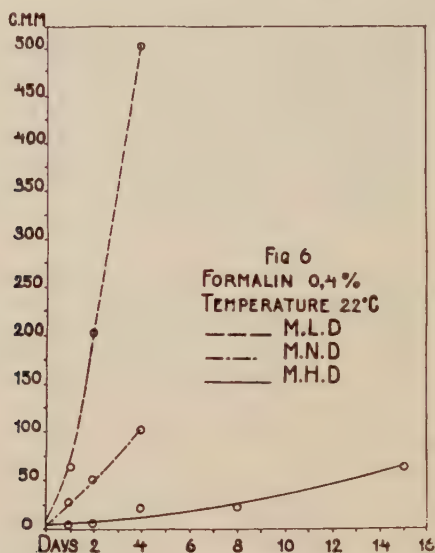


Fig. 6.

curve does not run steeply, whereas the lethal curve runs much more steeply than with 0.25 percent of formalin.

A study of the results presented in fig. 3 to 6 shows, that the lethal and necrotic activities are more readily damaged than the haemolytic. The addition of 0.25 percent of formalin and incubation at 22° C. seriously affects the first two properties. When the temperature of incubation is 37° C. however the effect is greater. The haemolytic power does not suffer a quick or considerable loss under these circumstances. Only when both the temperature and the content of formalin are unfavourable (fig. 4), does haemolytic activity disappear rapidly.

Summary.

The experiments show, that under certain well-defined circumstances both the lethal and the necrotic potencies of staphylococcal α -toxin can be destroyed, without much damage to the haemolytic power. After 3 days incubation at 37° C. with 0.25 percent of formalin, the lethal activity disappears, whereas the haemolytic potency remains high. The

same applies for an incubation period of 4 days at 22° C. with 0.4 percent of formalin. Under these conditions it is possible to obtain a modified toxin showing solely haemolytic power. This would apparently not be possible if both the haemolytic and the lethal properties were caused by the same toxic principle. These findings, therefore, strongly support the view, that haemolytic and lethal powers are caused by two different constituents of the toxic filtrate.

The same findings obtain when necrotic and haemolytic activities are compared.

The experiments do not permit to answer the question whether lethal and necrotic activities are manifestations of one or of two constituents.

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ABSTRACTS

J. REURINK, Natuurlijke Agglutinine (Natürliche Agglutinine). Diss. Amsterdam 1941, Verlag Scheltema en Holkema, Amsterdam.

Während man im Allgemeinen annimmt, dass die Bildung der Immunkörper statt findet infolge des Eindringens von Bakterien oder Bakterienstoffen in den Organismus, herrscht über den Ursprung der natürlichen Antikörper keine Uebereinstimmung. Hauptsächlich lassen sich über das Entstehen dieser natürlichen Antikörper zwei Theorien unterscheiden. Aus der Theorie der sogenannten „physiologischen Reifung“ erfolgt, dass die Bildung der natürlichen Antikörper eine durch Erbllichkeit im Laufe der Phylogenie erworbene Eigenschaft ist, welche allmählich nach der Geburt zur Entwicklung kommt. Der anderen Auffassung gemäss entwickeln sich die natürlichen Antikörper allmählich nach der Geburt in Antwort auf die Aufnahme bakterieller Antigene, welche von Saprophyten oder Kommensalen herkommen. Verfasser hat versucht mittels Versuche beim Huhn etwas zur Kenntnis der natürlichen Agglutinine bei zu tragen. Er konnte feststellen, dass das normale Hühnerserum imstande ist eine grosse Anzahl Bakterien verschiedener Art zu agglutinieren, während im Serum junggeborener Kücklein gar keine Agglutinine vorhanden sind. Letztere kommen sehr bald nach der Geburt zur Entwicklung. Die Art der Ausflockung und das Verhalten bei Erhitzung deuten darauf hin, dass die natürlichen Agglutinine bei Hühnern dem O-Typus gehören. Mittelst Absorptionsversuche konnte Verfasser feststellen, dass diese Agglutinine sehr spezifisch sind. Es war möglich aus dem Darm gesunder Hühner eine Bakterie zu züchten, welche stark agglutinierte mit normalem Hühnerserum. Es stellte sich heraus, dass diese Bakterienart (*Escherichia anindologenes*) gemeinsame antigene Eigenschaften besitzt mit *Shigella sonnei*, welche Bakterienart durch normales Hühnerserum ebenfalls stark agglutiniert wird. Obwohl die Ergebnisse dieser Untersuchungen Verfasser nicht zur Ablehnung der Reifungstheorie berechtigen, weist er darauf hin, dass dieselbe in Zusammenhang mit dem bakteriellen Charakter der natürlichen Agglutinine (Charakter der Ausflockung, Thermolabilität und Spezifität) mit der Theorie der aktiven Bildung der natürlichen Agglutinine nicht im Widerspruch sind.

AUTOREFERAT.

Papers presented in the 70th meeting of the „Vereeniging voor Waterleidingsbelangen in Nederland” (Netherlands Waterworks Association) in May 1941, published in Water **25**, 138, 153 and 163, 1941.

L. H. LOUWE KOOYMANS (State Institute of Water Supply), The significance of biological research in waterworks. As main problems he mentions:

1. The growth of algae and the prevention of their mass development. Plants purifying either surface water or deep well water in open filters are liable to such a development. The trouble caused by „water flower” in filters was mentioned and the offensive taste and odour provoked by the decaying microbes. Biological investigations into the factors influencing algal development might further the finding of successful measures.

2. Trouble caused by insects and other animal groups in the purification plant. The mosquito *Orthocladius perennis*, the caddice-fly *Neureclipsis bimaculata*, the polyp *Cordylophora caspia* and the mussel *Dreissensia* have caused serious trouble in waterworks of Berlin as well of Wiental.

3. The occurrence and development of higher organisms in the distribution system. Larvae of *Chironomus*, *Cyclops*, *Gammarus pulex*, *Nauplius* larvae, *Asellus* and *Nais* are of chief occurrence.

4. Biology of slow sand filtration. The earlier view that a filtering sand layer would act as a straining device for bacteria only when undisturbed, has been abandoned by more recent workers. A theory has been offered, which ascribed the filtering effect to the adhesive action of a layer of water coating the sand grains; this conception however has been refuted by HEYMANN. KISSKALT and SARDJITO claim that protozoa are the chief operators. Recently STUNDL has claimed the mechanical action of the filter to be the main factor. A wide field of research is offered to the biologist, although initial results will more probable occur in the scientific field than bearing on the technology of water works.

A. VAN DER WERFF (State Institute of Water Supply), The various organisms, significant for water works. He laid stress on the fact, that only a more detailed knowledge of the biology of these organisms might furnish a rational base for control measures. A survey of plants and animals, liable to be met with in water works and distribution systems was offered. The covering of reservoirs and filters was advised as a prophylactical measure and he claimed that the harmlessness of the

occurring organisms for the water consument was by no means proved.

In the discussion of both papers C. A. H. VON WOLZOGEN KÜHR (Water Supply of N. Holland) stated that a covering of filters would often be technically impossible and that an eradication of algae would often preclude the aerobic purification in the reservoirs. Organic matter occurring in the water offers favorable conditions for the development of higher organisms; merely bacteria, however, can mineralize all organic substance, but the process is too slow to be actually realized. This fact may hamper any biological control measures. The retaining effect of the filter is due to a slimy layer of organic substance, whereupon bacteria thrive. When pathogenic bacteria will occur in excessive numbers, they may not be retained completely. Normally, however, protozoa will keep down bacterial numbers on a stationary level. Up till now the higher organisms present are not known to have caused any deleterious effect on the water consumer, in contradistinction to the effect of bacteria. The need for biological research is not as pressing as that for bacteriological research in water works, although it may prove of value in the control of organisms causing technical trouble.

T. FOLPMERS (Rotterdam Water Supply) argues that a cleaning of filters with regular intervals precludes the occurrence of many groups of animals. In Rotterdam algal masses are retained by the Paterson pre-filters. A natural development of Crustaceae may preclude algal growth. The supply of cupric sulphate may entail the killing of Protozoa and Crustaceae, both of which are purifiers. The removal of a large amount of organic material is realized by means of a twofold filtration through sand layers of great depth, the sand bed as a whole contributing to the biological purification. According to speaker protozoa occur in large numbers in the superficial layers of sand filters. A covering up of filters is deemed undesirable because: 1) the light would not act in the killing of pathogens; 2) the stimulation of Protozoa to consume bacteria would be hampered; 3) development of sessile algae, which favour the action of the filter by the production of O_2 would be disturbed; 5) a higher content of CO_2 would render the water more aggressive.

Experiments with a small covered experimental filter applied after prefiltration have furnished proof of the occurrence of increased permanganate figures in the filtered water.

G. P. H. VAN HEUSDEN (Amsterdam Water Supply) mentions that in „green filters” anaerobic conditions may occur; the intensive production of organic matter may surcharge the filters and by withdrawal of CO_2 from the water $CaCO_3$ may precipitate and the filters get clogged.

It may even happen that algae pass the filter. Collected data indicate that temperature and light are of chief importance; the phenomenon may be foretold from weather conditions. *Daphnia magna* is known as a good destroyer of algae and is used as means of control. A solution of *Pyrethrum* is experimented with as means of control of *Asellus*.
T. F.

F. C. GERRETSEN en N. BLUMENDAL, Een onderzoek naar de bruikbaarheid van de *Aspergillus*-methode voor de bepaling van phosphorzuur en kali in den grond (An investigation as to the suitability of the *Aspergillus* method for the determination of phosphoric acid and potassium in the soil). Verslagen van Landbouwkundige Onderzoekingen, No. 46 (4) A, 219, 1940. Rijkslandbouwproefstation te Groningen.

An extensive methodical work. As the determination of phosphate has been treated by the authors in Antonie van Leeuwenhoek 6, 71, 1940 merely the determination of potassium will be summarized. The composition of a nutrient solution allowing an optimal development of *Aspergillus* was studied and the solution of NIKLAS had to be altered in several respects. Addition of Na was needed, even in the presence of much K. The addition of SiO_2 appeared as superfluous. A stronger buffering of the nutrient solution and a higher initial pH (3.5 instead of 2.4) acted quite favourably. The newly isolated strain „Groningen” was found superior to strain POSCHENRIEDER and BAYENS.

A large number of determinations was carried out with the improved method and the results were compared with several chemical extraction methods, with the method of NEUBAUER, and with field trials. The *Aspergillus* method correlated well with the results in the field, for the determination of phosphate as well as of potassium, so that it is justly recommended by the authors as a suitable method for serial work.

An interpretation for practical use of the figures obtained was worked out. The correspondence with other laboratory methods was more or less satisfactory.

A calculation of the costs of the *Aspergillus* method in serial determinations proved this method to contrast quite favourably with the usual chemical methods, whilst the accuracy and the reliability proved also in its favour. By introducing some suggested simplifications, especially by substituting estimation of the grown mycelia at sight, for their gravimetric estimation, which allowed of a quite satisfactory accuracy, the favourable relation could still be increased strongly.

H.

F. C. GERRETSEN, Iets over de omzetting van stalmest door micro-organismen (The decomposition of stable manure by microbes). Korte mededeeling van het Rijkslandbouwproefstation te Groningen.

This short paper is a compilation of modern empiric-practical literature on this subject and meant for use by the practical farmers. The decomposition of stable manure and other organic matter is discussed; changes in the C : N ratio are treated more in detail. Some directions are offered for the use of organic manure and for the treatment of stable manure during compostation. The differences between the resistant true „humus” and the easily decomposed organic manure is mentioned briefly.

H.

O. J. CLEVERINGA, De beteekenis van organische bemesting voor de instandhouding van de vruchtbaarheid van bouwgronden (The significance of organic manure for the preservation of the fertility of the arable soils). Special publication of the Geldersch-Overijsselsche Maatschappij van Landbouw, 1941.

A paper treating the specific value of organic manure for the structure and adsorption capacity of the soil, meant for practical farmers. The function of the micro-organisms in the decomposition of the organic matter in soil and the dung-pit is briefly discussed. The changes of the C : N ratio in manure and humus during decomposition and the formation of the resistant „true” humus are treated somewhat more extensively. The special importance of the C : N ratio of 20 : 1 and its action on the composition of stable manure are explained in a popular way. The structural condition and the supply with organic manure of the arable soils in the Netherlands are discussed and claimed to be on the whole in a very unsatisfactory condition.

H.

„Marsh-gas”, Redactional paper in Water, Bodem en Lucht **31**, 87, 93, 1941.

A critical article treating the paper of J. G. DE VOOGD, Het Gas **61**, 213, 225, 1941. Several data on the number and distribution of gas-wells in the Netherlands and on the gas derived from them are gathered. The disadvantages of the thus gained gas, such as the pollution and the increase of the chloride content of ditches and canals and the costs of extra draining are discussed. The origin of marsh-gas is treated and its composition is compared with that of gas derived from sewage by the activated sludge-method. The microbiology of the process is hardly discussed.

H.

(From the Biochemical Institute of the State University of Leiden and from the State Institute for Public Health at Utrecht).

THE ELECTROPHORETIC BEHAVIOUR OF *BACTERIUM TYPHOSUM* IN RELATION TO THE CHANGES OF THE ANTIGENIC STRUCTURE OBSERVED IN THE SMOOTH-ROUGH VARIATION

by

W. A. L. DEKKER, C. VAN DER MEER AND R. TH. SCHOLTENS

(Received March 24, 1942).

1. INTRODUCTION.

During the last years the relation between the electrophoretic behaviour and cultural appearances of smooth and rough cultures of bacteria has been subject of study.

Mme SORU (17) — in *Vibrio cholerae* —, Mlle CHOUCROUN (5) — in tubercle bacteria —, and Miss JOFFE and MUDD (9) — in *Bacterium typhosum*, *coli*, *flexneri*, *enteritidis* and *pullorum* — showed that the rough forms possess a higher negative charge than the smooth forms under the same experimental conditions.

Working on the mechanism of immunity reactions DEKKER suggested with regard to the possible protein nature of the antigens to characterise these substances by means of their electrophoretic behaviour and their iso-electric point. Therefore, in collaboration with C. VAN DER MEER and H. BEEUWKES, the electrophoretic velocities of various bacteria (*B. shigae*, *Staphylococcus albus* and *B. pseudotuberculosis rodentium*) were determined at different pH values. In these investigations (unpublished) differences between the rough and smooth forms similar to those found by the above mentioned authors were established. It was evident, however, that a distinction between R and S forms by mere cultural characteristics did not suffice to explain all types of pH-electrophoretic velocity curves obtained.

SCHOLTENS, working in the same period with *Bacterium typhosum*, showed that the stability of bacterial suspensions in salt solution depends on the presence of Vi or other O antigens (14, 15), whilst the zones of acid

agglutination (in buffer mixtures) depend on the presence of the H antigen or the Vi antigen respectively (16). From these facts he concluded that a relation exists between antigenic structure and physico-chemical characteristics of micro-organisms.

We decided to investigate how far the curves, obtained by plotting the electrophoretic velocities of various strains of *Bacterium typhosum* as a function of pH, show variations parallel to those in antigenic structure, viz., whether the special antigens found on the surface of the bacterium alter the shape of these curves.

A few observations on the antigenic structure of *Bacterium typhosum* should precede the description of the experimental part.

2. THE ANTIGENIC STRUCTURE OF *Bacterium typhosum* AND ITS VARIATIONS.

The antigens of *Bacterium typhosum* are:

I. The thermolabile H antigen, which is localised in the flagellae.

II. The thermostable O antigen complex. This antigen complex is localised on the surface of the bacteria. WHITE (19) gives the following definition of the smooth-rough variation, which is dominated by the O antigen complex: It is „a change, sometimes reversible, perhaps, when complete, irreversible, of the stable (O) complex of the organism into a serologically different complex (Ø), which is debased in specificity. This change named with reference to the cultural modification with which it is associated is the smoothform → roughform variation of ARKWRIGHT". The above definition implies, that the cultural smoothform → roughform modification (loss of stability in salt solution and of smooth colony form i.e. growing autoagglutinable and acquiring a rough colony form) being associated with the loss of the O antigen complex is a characteristic of the O antigen complex. When discussing the Vi antigen this point will be referred to in detail.

The O antigen complex of *Bacterium typhosum* consists of the following antigens.

a) The IX antigen. This antigen is thus mentioned in the KAUFFMANN-WHITE scheme. In normally prepared Gärtner and Typhoid sera there is in most cases a comparatively large agglutinin fraction (viz., 1/800 to 1/6400) which corresponds with this antigen.

b) The (XII) antigen. The antigen indicated thus by KAUFFMANN (11) does not occur in the KAUFFMANN-WHITE scheme, as serologically it has not been fully examined. KAUFFMANN states the following with regard

to this antigen: „Es handelt sich hier um ein 0 antigen, dass den Typhus, Stanley, Reading und anderen Typen gemeinsam ist. Dieser Faktor ist jedoch auf Grund von Untersuchungen von P. B. WHITE complex gebaut, und nicht in allen diesen Typen gleichmässig vorhanden”.

c) The Vi antigen This antigen was discovered by FELIX (6). He was able to prove, that the presence of this antigen in strains of *Bacterium typhosum* increased their degree of virulence (intraperitoneal injection in mice). For the following reasons, from an immunological viewpoint he has placed this antigen in a separate class.

According to FELIX this antigen could only increase the virulence induced by the 0 antigen¹⁾. In the absence of the 0 antigen the Vi antigen therefore could not affect the virulence at all. Consequently he reported that strains having the Vi antigen, but no 0 antigen, were avirulent (FELIX and PITT (7)). Now in respect of virulence of strains possessing the Vi antigen but no antigen IX (XII) our experience differs to some extent from that of FELIX. SCHOLTENS and POT (unpublished) have found, that besides strain Ty 4516 described by POT and DORNICKX (12) also strain Ty 359 which had not any 0 antigens similar to those of Gärtner bacteria either, was decidedly virulent. (POT and DORNICKX mentioned strain Ty 4516 as 0-resistant. FELIX understood 0 resistance as meaning that a strain cannot be influenced by 0 agglutinins (IX (XII)) despite the presence of these antigens in the bacteria. In our opinion to consider the strain Ty 4516 as 0 resistant, because it neither possesses the antigens IX nor (XII) as POT and DORNICKX did, was incorrect).

The same quantity of these strains Ty 4516 and Ty 359 was sufficient to kill a mouse by intraperitoneal injection as in the case of other strains containing both the Vi antigen and the antigen IX (XII). This proves that this antigen does not (as FELIX indicated) merely increase the virulence induced by the 0 antigen (here IX (XII)), but also independently can turn a strain virulent for mice. Although this antigen increases the virulence there is no reason to assume that here we have a feature, which immunologically would cause this antigen to be placed in a different class. A difference can not be shown between the way in which the Vi antigen increases the virulence and the manner in which this is generally done by 0 antigens. The activity to increase virulence therefore is

¹⁾ In FELIX publications the 0 antigen of *Bacterium typhosum* appears to be a thermostable antigen complex, causing granular agglutination, which these bacteria have in common with Gärtner bacteria. This antigen complex consists of the antigens IX and (XII).

a characteristic which the Vi antigen has in common with 0 antigens and this would account for its being classed as an 0 antigen.

KAUFFMANN (11) in his study on typhoid antigens considered the Vi antigen as the only example, so far, of a group of antigens distinguished by their being body antigens and losing their agglutinogenic and antigenic properties after two hours at 100° C. BOIVIN (2) showed, however, that the Vi antigen in his bacterial extracts did not lose these properties after two hours at 100° C. and (1) that the Vi and IX antigens chemically were of similar description. He classified the Vi antigen among the 0 antigens (2). This opinion agrees with the observations of SCHOLTENS (14) who — too — placed the Vi antigen on one line with the 0 antigens. He described a parallelism as existing between the antigenic structure and the cultural appearances of *Bacterium typhosum*, as follows:

Strains, containing both the Vi antigen and the antigen IX were stable in salt solution. The colony form was typically smooth. The strains, which contained one of the two antigens, antigen IX or the Vi antigen, were also stable in salt solution. Their colony form was often perfectly smooth, at times slightly irregular and on the rough side, but never typically rough. Only those strains, which contained neither the Vi antigen nor the antigen IX were auto-agglutinable and showed, without exception, a typically rough colony form.

It is clear from the above, that, in the case of *Bacterium typhosum* the cultural modification from smooth to rough can be associated with both the loss of the Vi antigen and with the loss of the IX (XII) antigens. The association of the cultural smooth-rough variation with the loss of the Vi antigen could be studied most effectively by observing the variation of the strains containing Vi antigen and no IX (XII) antigens under the influence of Vi bacteriophages. The association of this cultural modification with the loss of the Vi antigen is a further characteristic which this antigen has in common with the 0 antigen (see P. B. WHITE, quoted above), and is one more reason for ranking it among the 0 antigens.

SCHOLTENS (15) drew the following diagram for the smooth-rough variation of *Bacterium typhosum*¹⁾:

¹⁾ In the publication in which this scheme was originally published (15), the term „0 antigen” takes the place of the cipher IX (See also FELIX contention stated above). Now that it is stated that the Vi antigen also belongs to the 0 antigen complex of *Bacterium typhosum* the term 0 antigen cannot be used in this sense.

Smooth form			
IX,	Vi,	(H)	
IX-Halfsmooth form		Vi-Halfsmooth form	
— IX,	(H)	— Vi,	(H)
Rough form			
— —	(H)		

An exact serological analysis of (XII) antigens lacking, it is not possible to define their complete significance for the S-R-variation. Hence the above scheme does not mention the (XII) antigens. There were no deviations of the parallelism between the antigenic structure and the cultural smooth and rough properties, described above, which had to be ascribed to the (XII) antigen. The possibility is not excluded, however, that such deviations would occur.

We have compared the antigenic structure of the variants occurring in this scheme with their electrophoretic behaviour whereby specially the presence or absence of H, IX and Vi antigen was taken into account.

3. TECHNIQUE.

I. Preparation of the bacterial suspensions.

A Roux bottle with broth agar medium was inoculated with the strain under examination, and kept at 37° C. overnight. The culture formed was suspended in distilled water, and centrifuged. After decanting the supernatant fluid this procedure was repeated. Now the sediment was suspended in 20 cc of distilled water. With part of this suspension the antigenic structure of the bacteria was determined. The rest of the suspension was heated in a sealed ampule for 15 minutes in a boiling waterbath.

For the electrophoretic examination a 1 : 10 dilution was made by adding to 10 cc of the heated suspension 10 cc 1 N NaCl solution and 80 cc of distilled water. The addition of NaCl is not essential but it facilitates the determination of the potential gradient in the measuring cell (to be described below) by improving the conductivity of the medium. To 45 cc of solutions of hydrochloric acid of varying concentrations 5 cc of the diluted bacterial suspension were added. In this way, while a NaCl concentration of 10 milliequivalents was kept constant, the acidity of the dispersion medium was varied between pH = 0 and pH = 6, without using buffer mixtures, the ions of which might influence the charge of the suspended particles considerably and differently.

II. Determination of the antigenic structure.

To indicate the presence of the Vi and H antigen, adsorption tests were made as described on a previous occasion by SCHOLTEMS (14, 15). The presence of IX agglutinine was established by indicating, that the typhoid strain under examination caused a fall in the titre of a Gärtner serum towards the typhoid strain O-901 (IX, (XII)). As this typhoid strain O-901 is agglutinated by the anti-IX and anti-(XII) bodies this fall of the titre must be caused by an adsorption of the anti-IX and/or (XII) agglutinins. That this fall of titre does not occur by adsorption of (XII) agglutinins without adsorption of the IX agglutinin may be concluded from the fact that adsorption of the (XII) fraction by means of a mixed suspension, prepared with cultures of strains of *Bacterium* Pty A (a.o. Pty A Durazzo), Aertrycke, Stanley, Reading and Schottmüller, did not cause a lowering of the titre of this serum for the typhoid strain O-901.

If it appeared, that the strain examined in this adsorption test did not lower the titre of the Gärtner serum for strain O-901, then a further test was made, to make sure, that no adsorption of IX agglutinins was masked by the (XII) agglutinins. An adsorption test was made with the same Gärtner serum from which now the (XII) agglutinins were removed by a previous adsorption with the mixed suspension as described containing (XII) antigen. In none of the cases in which in the former adsorption test no lowering of the titre was observed, such a lowering was found in the latter, more differentiated adsorption test. Thus it was concluded, that the strain under examination did not contain the IX antigen.

III. Determination of the pH.

Together with the measurements of the electrophoretic velocity in the above mentioned mixtures the hydrogen ion concentration was determined by means of a glass electrode.

IV. Determination of the electrophoretic velocity.

A. Apparatus.

For the determination of the electrophoretic velocity of the bacteria we used a micro-cell of the closed type constructed by DEKKER¹⁾ — a modification of the instrument described by BUNGENBERG DE JONG and

¹⁾ Though not yet published before, this micro-electrophoresis cell has several times proved its usefulness (see (3,10)).

TEUNISSEN (4) — the upper and side aspects of which are represented in the schemes 1 and 2.

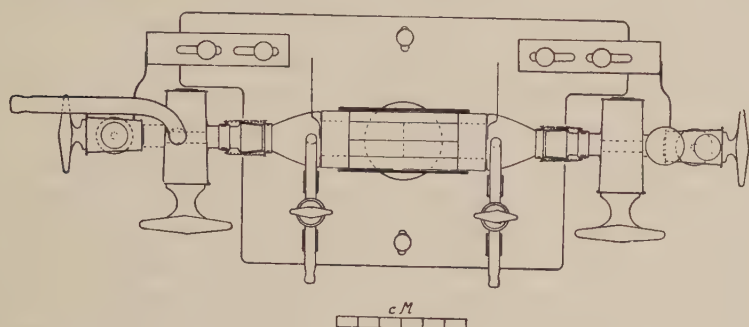


Fig. 1. Upper view of the micro electrophoresis cell.

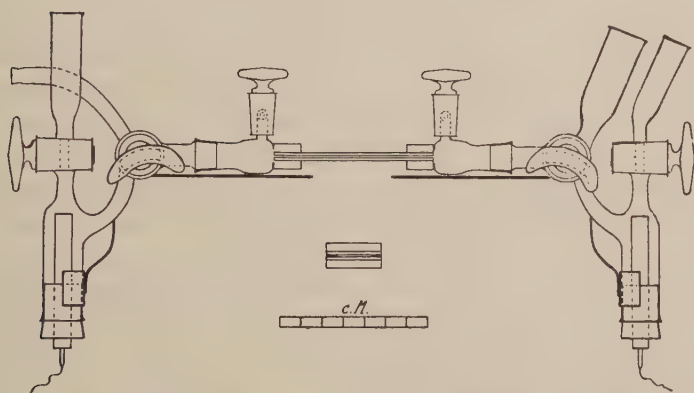


Fig. 2. Side view of the micro electrophoresis cell.

This electrophoresis cell consists of three separate parts, *viz.*, a central part and two side pieces, which may be united to a closed system by means of standard ground joints. To prevent leaking these latter are carefully lubricated and strongly compressed by metal springs.

In the central part may be distinguished:

a) The actual measuring chamber, a narrow, flat space (length 76 mm, width 18 mm, thickness 0.8 mm) bordered by planoparallel walls, made of ground object glasses and small bars of glass, and held together with „Khotinsky” kit. In order to facilitate the determination of the height of the flat space (necessary for the measurement of the electrophoretic velocity, see below) the inner sides of upper and lower walls have been

marked with fine scratches. Care must be taken that the point where the two scratch-marks cross each other (about halfway the length of the measuring chamber) is situated in the periphery of the visual field of the microscope.

b) Glass cups. These are fixed with „Khotinsky” to the ends of the measuring chamber and are provided in their highest points with capillaries fitted with well-ground and lubricated stop cocks.

These capillaries are connected by two rubber tubes (not indicated in the schemes) with a Y-shaped glass junction from which a third tube leads to a collecting vessel, so that air bubbles can be easily removed from the apparatus. Just below the entrance to the measuring chamber the glass cups are fitted with sealed-in platinum wires, by means of which the potential gradient in the actual measuring space can be determined potentiometrically ¹⁾.

The two side pieces contain the unpolarizable electrodes (bars of pure zinc immersed in a solution of the following composition ²⁾: 200 g of $\text{CaCl}_2 \cdot 6\text{aq}$ + 20 g of ZnCl_2 + 100 cc of distilled water) between which by means of an anode-accumulator an electromotive force of 100 volt is applied. They are provided with well-ground and lubricated three-way stop cocks, which make the communication between measuring chamber and zinc electrode (I) or between the former and the supply respectively outlet (II) and which are constructed in such a way that in communication I the full opening of the cock is turned towards the measuring space but the opposite opening is only partly in contact with the electrode liquid (owing to which fact the diffusion of Zn- and other ions towards the middle piece is highly limited), whereas in communication II the two openings of the cock are fully used.

Right above each zinc bar lies a glass cup, separated by a stop cock from the actual electrode-space; it forms a reservoir from which the electrode liquid may be replenished and at the same time an outlet for the gas bubbles produced by the electrode. Left and right side piece only differ by the fact that the latter possesses a second cup, by which washing-liquid or suspensions under examination are supplied while the

¹⁾ The sealed-in platinum electrodes have not been placed in the measuring chamber itself, since gas bubbles interfere more strongly in the narrow space than in the glass cups; also the cleaning of the middle piece would be more difficult.

²⁾ The indicated electrode liquid has the advantage over the commonly used zinc sulphate solution that precipitates (e.g. in the presence of Ba- or Ca-ions) which might clog the openings of the stop cocks occur less frequently.

former is provided with an outlet tube through which the content of the micro-cell may be removed.

The micro-cell is fixed upon a brass plate (with an opening for the microscope condensor to pass), the side pieces being supported by copper brackets which can be screwed upon this plate. The whole has been mounted upon a Zeiss microscope stand ESC (instead of the usual square table), provided with an oblique eyepiece (Monokni E). The suspended particles (bacteria, etc.), the rate of migration of which is to be determined in the electric field, are observed under a magnification of $280 \times$ (apochromatic objective $10 \times$, orthoscopic eyepiece $28 \times$, in which an ocular micrometer 5 : 50) on a dark ground obtained by means of a Zeiss „Präparier Wechsel“-condensor n.a. 0.7–0.8 and a Zeiss low-voltage microscope lamp as a source of light. Supplementary instruments are:

1. An anode-accumulator of 100 volt, which during the measurement of the electrophoretic velocity is constantly charged by means of a rectifier connected with the lighting circuit in order to keep the potential difference constant, its voltage being checked with a voltmeter (0–120 volt).

2. A potentiometer, composed of an anode-accumulator of 60 volt, variable resistances (two radio resistances of 5000 and 400 ohms respectively), a simple needle galvanometer (e.g. Pye's), a precision voltmeter (measuring range 0–120 volt, readable in 0.1 volts), a spring commutator, a 10000 ohm protecting resistance, a push-button.

3. A stop-watch, readable in 0.2 seconds.

4. A spring commutator fixed upon the work-bench, by which the electric field may be reversed.

5. A bottle placed on a stand about 1 metre above table level, from which distilled water through a rubber tube provided with a clamp can be led to the cell in order to rinse the apparatus.

6. A three-way stop cock fixed upon the work-bench and connected with rubber tubes *a*) with the outlet tube of the micro-cell, *b*) with an aspirator and *c*) with a sink. It enables on the one hand to clean the cell by sucking a powerful stream of water through the measuring chamber and on the other hand to fill the apparatus slowly and gradually.

B. Measurements.

For determination of the electrophoretic velocity it is not indifferent at which level in the measuring chamber the rate of migration is observed. As a matter of fact, under the influence of the applied e.m.f. a water

transport (electroendosmosis) occurs along the charged glass walls. In a cell of infinite width the effect of the sides may be disregarded and one would have only to take account of the displacements of liquid along the upper and lower wall, both flowing in the same direction. However, in a space closed on all sides the transported water must flow back again, which it does through the middle of the cell. So three moving liquid layers arise, the two electroendosmotic currents along the upper and lower wall in one direction, and the pressure current through the middle in the opposite direction. Between these moving layers there are two stagnant ones. The migration velocities of the suspended particles lying in these two layers are due to their electric charges only and are not influenced by electroendosmosis.

VON SMOLUCHOWSKI calculated that in a flat cell of infinite width the two stagnant layers are found at distances $1/5$ th and $4/5$ th of the total distance from the upper and lower wall. It has been demonstrated by ALLEN and others (see VAN GILS (8)), that the relation derived by VON SMOLUCHOWSKI is already applicable when the ratio of width to height of the measuring chamber is larger than 5, which condition has been fulfilled in the above-mentioned electrophoresis cell.

In order to determine the height of the flat space the cell is completely filled with liquid. Then the micrometer screw of the microscope (provided with a scale division and turned down as far as possible) is set at the zero mark, whereupon the microscope by means of the coarse screw is focussed on the edge of the fine scratch mark made upon the inside of the lower glass wall. Subsequently the tube of the microscope is turned up so far by means of the micrometer screw that the edge of the scratch mark made in the upper wall is sharply observed: the number of rotations of the micrometer screw is now a measure for the height of the flat space. To focus the microscope on one of the stagnant liquid layers (e.g. at $1/5$ th of the height of the measuring chamber beneath the upper wall) the micrometer screw is turned back over the calculated number of rotations.

When the cell is filled ¹⁾ with the bacterial suspension, the actual

¹⁾ After careful removal of the preceding content and of the penetrated electrode liquid (for which purpose a powerful stream of air and water is sucked with the aspirator through the cell) the apparatus is rinsed 5 times with each time 5 cc of the suspension under examination (with simultaneous removal of all air bubbles through the capillaries of the mid-piece), the last portion at the same time forming the filling of the micro-cell on which the measurements are made.

measuring of the electrophoretic velocity consists in determining with a stop-watch the time (t_1) required by a bacterium (lying in the stagnant liquid layer) to cover a certain distance (e.g. of 5 marks ¹⁾ of the ocular micrometer) after a known e.m.f. is applied. Immediately afterwards the electric field is reversed by means of the spring commutator fastened on the work-bench and again the time (t_2) is measured in which a bacterium in an opposite direction covers a same distance. The obtained times are recorded in pairs and provided with a — or + (indicating the sign of charge of the observed bacteria, judged by the direction in which they move in the visual field). In order to facilitate the identification of the sign of the electric charge, the circuit has been arranged in such a way that positively charged particles in the visual field move in the direction to which the handle of the commutator points.

12 pairs of measurements ²⁾ are made on one and the same suspension. In order to obtain the electrophoretic velocity we must not calculate the average of the recorded times but that of their reciprocals: for this purpose the values $1000/t_1$ and $1000/t_2$ are looked up in a table of reciprocals and added to each other, so that influences of possible convection currents ³⁾ are eliminated, and of the 12 figures obtained in that way the average is determined, which result consequently represents 2000 times the average electrophoretic velocity (expressed in completely arbitrary units); in order to indicate it in an absolute measure, i.e. in μ per second at a potential gradient of 1 volt per cm, the real distance covered by the bacteria in the visual field in the recorded times has to be determined (with the aid of an object micrometer) as well as the potential gradient in the measuring chamber (practically between the

¹⁾ In the case of very small velocities the time required for covering a smaller distance, e.g. 1 mark of the ocular micrometer, is measured and then calculated for a distance of 5 marks.

²⁾ For strongly deviating values of the sum of the reciprocals (added in pairs) a larger number of measurements must be performed; however, a restriction for the extension of the number of measurements is formed by the diffusion of strongly discharging ions from the electrode liquid; see note 1, page 64.

³⁾ Sometimes (mainly with small electrophoretic velocities) t_1 and t_2 have not the same sign of charge, at any rate judged by the displacement in the visual field; the reason for this is that an incidental convection current lends a greater migration velocity to the particles than that brought about by the potential gradient. However, by the indicated method of calculation (the algebraic summation of t_1 and t_2) the influence of these convection currents is eliminated.

platinum electrodes, whose mutual distance has to be measured). Since by slow diffusion of ions from the electrode liquid in the micro-cuvette ¹⁾ a uniformly changing potential gradient in the measuring space occurs, the potential difference between the platinum electrodes both at the beginning and at the end of the measurements has to be ascertained on the same system and from this an average tension must be derived.

4. THE ELECTROPHORETIC BEHAVIOUR OF ANTIGENIC VARIANTS OF *Bacterium typhosum* AT DIFFERENT pH.

The data, obtained in our study of the electrophoretic velocity of some 20 strains of *B. typhosum* as a function of the hydrogen ion concentration of their suspension media, are shown in the figures 3—7. In these graphs the pH has been plotted on the abscissae and the electrophoretic velocity *U* (expressed in arbitrary units, 1000 of which correspond to an absolute velocity of 1.27μ /sec. per volt/cm) on the ordinates; also the presence or absence of the 0 antigens IX and Vi, as determined serologically in the living bacteria, is indicated.

The curves resulting from these experiments give rise to the following conclusions:

I. The H-antigen, if present in the living bacteria, has no influence upon the charge of the heated bacteria, a fact which agrees with the thermolability of this antigen. (Compare in fig. 5 strain O-901 with the other IX-halfsmooth forms and in fig. 6 strain S 44111 with the other Vi-halfsmooth forms).

II. By means of the pH-*U*-curves four groups of *B. typhosum* can be identified (fig. 3, 4, 5 and 6), corresponding closely to the four types differentiated by serological methods. In other words, there is a definite correlation between the charge of the (heated) bacteria and the antigenic structure.

III. The curves of the rough bacteria (neither IX nor Vi present; see fig. 3) show iso-electric points between pH 2.0 and 2.5, thus indicating the amphoteric nature of the 0 antigens.

IV. By comparing the curves of the R bacteria with those of strains having Vi antigen but no IX (fig. 6), and those of the S forms (having

¹⁾ The diffusion of ions from the electrode liquid towards the measuring space is the reason why the total duration of the measurements on one suspension may not exceed a time of c. 25—30 minutes, since the charge of the suspended particles otherwise is influenced as well by the Zn- and Ca-ions.

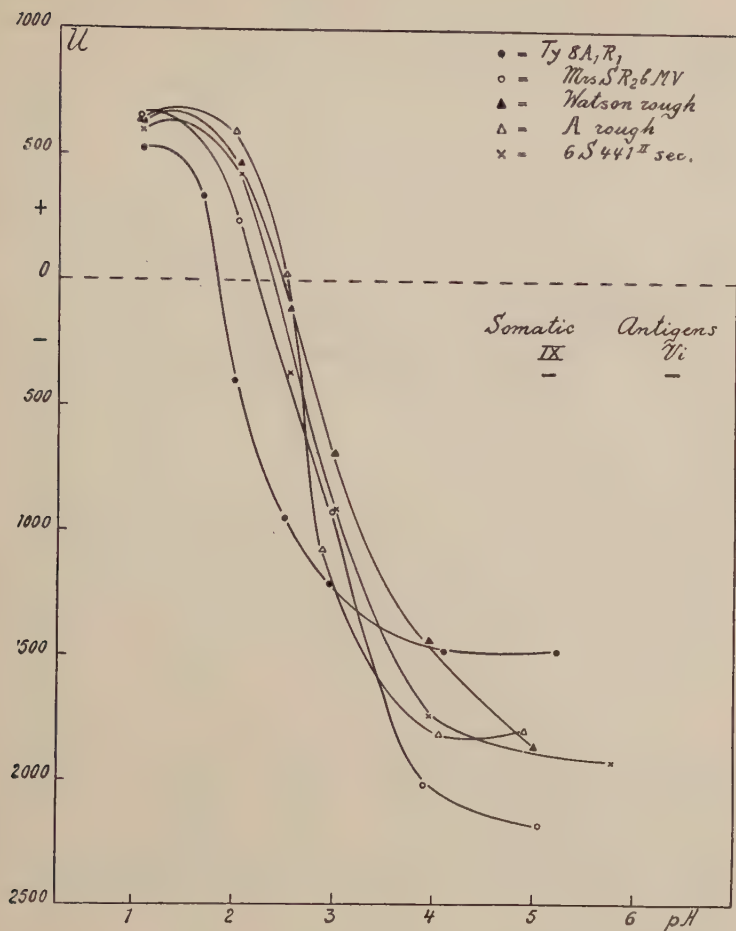


Fig. 3. The electrophoretic mobility, as a function of pH, of the R-forms of *B. typhosum*.

IX and Vi; fig. 4) with the curves of strains belonging to the IX-half-smooth type (with IX, but without Vi; fig. 5) the tendency of the Vi rest (resulting from the heating to 100° C. of this antigen) to induce a negative charge to the bacterial surface is clearly shown. As this negativating influence is even perceptible at a pH below 2, the ionogenic groups of this Vi rest probably are of the character of a strong acid (sulfuric or phosphoric acid groups?).

V. The shape of the pH-U-curve of the smooth type (fig. 4) gives rise to the supposition, that the surface charge of these bacteria must

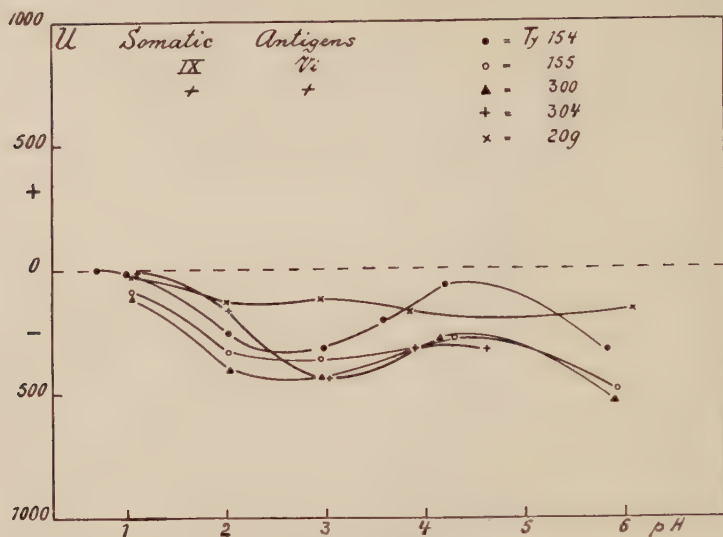


Fig. 4. The electrophoretic mobility, as a function of pH, of the S-forms of *B. typhosum*.

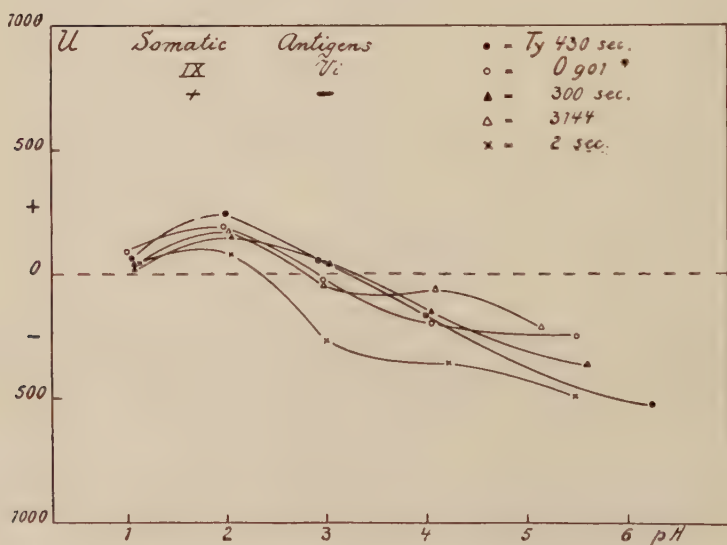


Fig. 5. The electrophoretic mobility, as a function of pH, of the IX-half-smooth forms of *B. typhosum*. * The living bacteria contained no H-antigen.

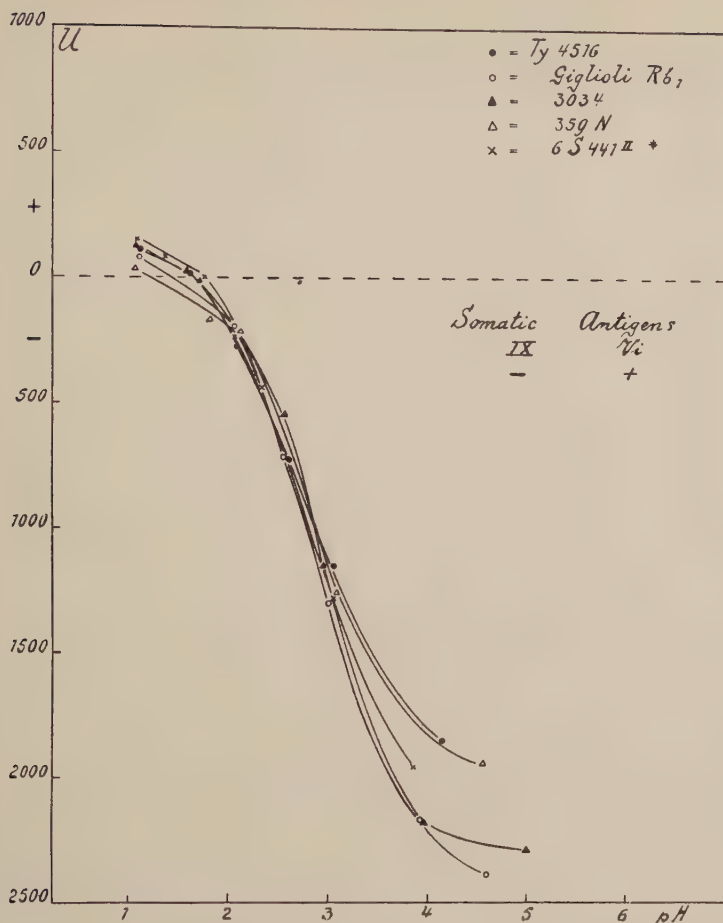


Fig. 6. The electrophoretic mobility, as a function of pH, of the Vi-half-smooth forms of *B. typhosum*. * The living bacteria contained no H-antigen.

be considered the algebraic sum of the negative charge induced by the Vi rest and the positive or negative charge (depending on the pH) of the amphoteric somatic antigen IX¹). The minimum in the electrophoretic curve at pH 4.5 would then correspond to a maximum positive charge

¹) This conception of a mosaic structure of the surface of *B. typhosum* is in accordance with the opinion of Por (13), who from agglutination experiments came to the conclusion that the smooth form (in his nomenclature the V form) must also possess O receptors (i.e. IX receptors) besides Vi receptors on their surfaces.

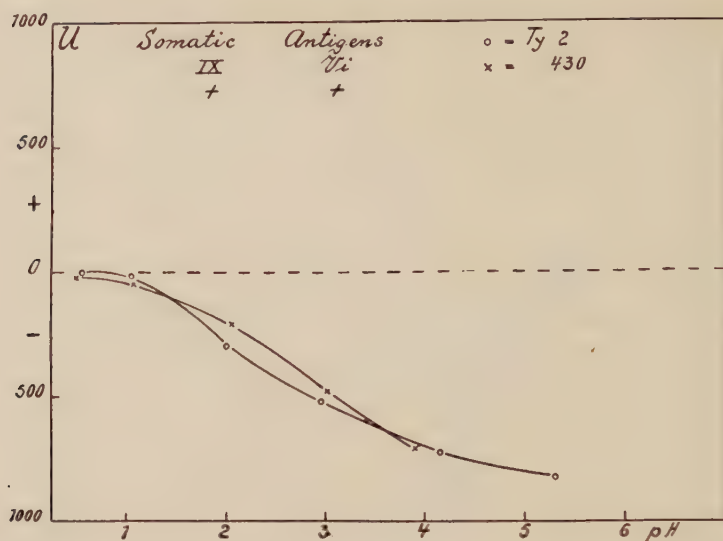


Fig. 7. The electrophoretic mobility, as a function of pH, of the strains Ty 2 and Ty 430 (S-forms) of *B. typhosum*.

of IX antigen, which implies an iso-electric point above pH 4.5 for the latter.

This is not at variance with the fact that in the case of strains, having the IX antigen but without Vi, an iso-electric point is found at about pH 3, which may be very satisfactorily explained on account of the imperfect covering of the bacterial surface by IX antigen, so that the charge of this type is determined by both IX and the θ antigens. The fairly large margin between the iso-electric points of the last mentioned intermediate forms agrees with this viewpoint (so we found a reversal of the charge at pH 2.3 in a secondary culture of the strain Ty 2, obtained by treatment with a Vi bacteriophage).

VI. A behaviour different from the other S forms is found in two additional strains Ty 2 and Ty 430 (see fig. 7) containing both IX and Vi antigen. We are inclined to ascribe the peculiar shape of their pH-electrophoresis curve to the presence of a great quantity of Vi antigen (the strain Ty 2 of FELIX is known to be very virulent for mice!). However, as precise knowledge of the virulence of the bacteria under examination is lacking, it would seem advisable to delay the expression of our opinion on this exceptional behaviour until further investigations will be made.

Following our electrophoretic experiments we are able to make a diagram of the arrangement of the body antigens on the surface of

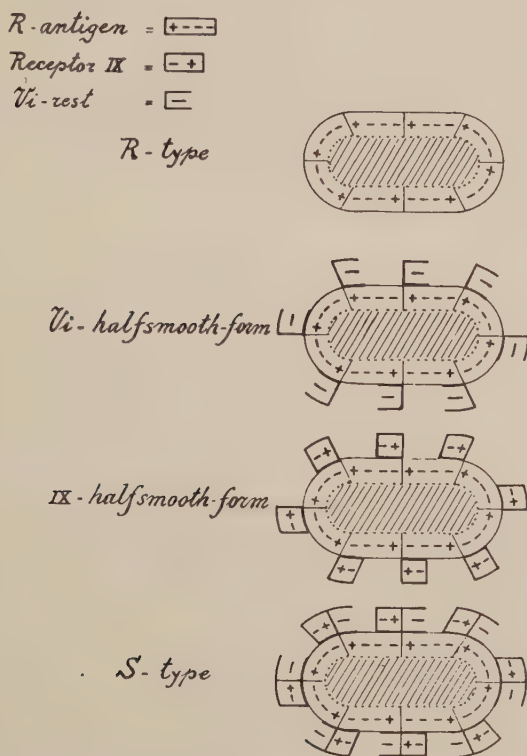


Fig. 8. Diagram of the body antigens on the surface of *B. typhosum*.

B. typhosum (see fig. 8) which is in agreement with the general pictures given by TOPLEY (18).

S u m m a r y.

I. Reasons are given for the classification of the Vi antigen among the 0 antigens.

II. A description is given of a micro electrophoresis cell with which measurements of the migration velocity of bacteria can be easily performed.

III. The pH-electrophoresis curves of different strains of *B. typhosum*, determined by the method described, leads to a classification in four different groups corresponding closely to four different antigenic types.

IV. Most likely the θ -antigens are amphoteric substances with iso-electric points between pH 2.0 and 2.5 (nucleo-proteins?).

V. After heating up to 100° C. the Vi antigen rest, even at pH < 2, possesses a negative charge. In all probability it contains groups strongly acid in character (phosphoric acid, sulphuric acid?).

VI. It is supposed that the somatic antigen IX is an amphoteric substance with an iso-electric point above pH 4.5.

VII. A provisional diagram of the localisation of the antigenic components on the bacterial surface is given.

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SOME OBSERVATIONS ON THE METABOLISM OF BACTERIA OXIDIZING MOLECULAR HYDROGEN

by

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(Received June 16, 1942).

1. BRIEF REVIEW OF PREVIOUS INVESTIGATIONS ON HYDROGEN OXIDIZING BACTERIA.

Already in 1839 DE SAUSSURE (1) observed that in the presence of soil a mixture of hydrogen and oxygen gradually disappeared and apparently was converted into water. In 1892 IMMENDORF (2) reinvestigated this phenomenon and observed along with other facts that addition of chloroform to the soil prevented the gas consumption. In this way the biological nature of the process had been made extremely probable.

Experimental proof that bacteria were responsible for the hydrogen oxidation was brought almost simultaneously by NIKLEWSKI (3) and by KASERER (4). The latter inoculated a purely mineral medium with some garden soil and found that on bringing this medium into contact with a gas mixture containing hydrogen, oxygen and carbon dioxide a bacterial film developed which actively oxidized the hydrogen. KASERER gave a description of the organism to which he gave the name of *Bacillus pantotrophus*. He failed, however, in his attempts to bring it into pure culture. NIKLEWSKI made more or less similar observations.

LEBEDEFF (5) was the first in claiming to have obtained a pure culture of a hydrogen oxidizing bacterium, but he does not give many particulars regarding the organism, nor regarding the procedure applied in its isolation. He made, however, many experiments regarding the ratio of the amounts of hydrogen and of oxygen consumed under different conditions. This ratio proved to be subject to rather considerable fluctuations. Since, however, LEBEDEFF also established that the nitrate present in his medium was reduced and was partly converted into elementary nitrogen, it seems likely that the evolution of this gas will have impaired in some measure the correctness of the analyses made.

In a continuation of his earlier studies NIKLEWSKI (6) reported the isolation of two different hydrogen oxidizing bacteria which he brought into the genus *Hydrogenomonas*, previously created by ORLA-JENSEN. NIKLEWSKI found that neither his *Hydrogenomonas vitrea*, nor his *Hydrogenomonas flava* were able to grow for themselves in the hydrogen-oxygen atmosphere used in the enrichment culture. This situation changed, however, when the oxygen tension in the atmosphere was lowered to 53 mm mercury. He also established the heterotrophic mode of life of both species by showing that they were able to grow in the absence of hydrogen if suitable organic substrates were made available. Such substrates prevented more or less completely the oxidation of hydrogen. In consequence hereof he emphasizes the importance of a further study of the mechanism of this oxidation for the obtaining of a better insight into respiration processes in general.

The results of a very thorough investigation of bacterial hydrogen oxidation have been published by RUHLAND (7) in 1924.

RUHLAND made a great number of experiments regarding the gaseous metabolism of growing cultures of *Bacillus pycnoticus*, a spore-forming rod which actively oxidized hydrogen. In the first place he could corroborate NIKLEWSKI's observation that addition of organic substrates, like glucose and mannitol, to the culture medium inhibits the oxidation of hydrogen. RUHLAND then very carefully determined the ratio between the amounts of hydrogen and oxygen consumed, and established that this ratio as a rule surpasses the theoretical value 2 quite considerably. Values between 2.78 and 2.14 were found. Moreover, RUHLAND could prove that the surplus of hydrogen consumed corresponded quite satisfactorily with the amount of carbon dioxide assimilated in a proportion of 2 : 1, which fact strongly indicates the carbohydrate nature of the assimilation product formed. In a few experiments in which the hydrogen oxidizing capacity of pre-formed bacteria were tested in the absence of carbon dioxide the hydrogen/oxygen ratio was determined as 1.80. Here apparently the assimilation process was practically excluded, whilst part of the oxygen present was used in the oxidation of cell material. We will postpone the discussion of the general conclusions drawn by RUHLAND from his experiments to the next chapter.

At about the same time a paper was published by RUHLAND's collaborator GROHMANN (8), in which paper the wide distribution of bacteria capable of hydrogen oxidation in nature was amply demonstrated. GROHMANN showed that this faculty is encountered amongst representatives of widely different natural groups of bacteria. This point of view

has since been confirmed by several other workers and forms a strong argument against the still prevailing tendency of uniting all hydrogen oxidizing bacteria in a separate genus: *Hydrogenomonas*.

Mention should be made also of GROHMANN's remarkable experience that after his removal from Tübingen to Leipzig all his cultures spontaneously lost their ability to bring about hydrogen oxidation, and of the even more startling fact that new attempts to obtain enrichment cultures remained unsuccessful.

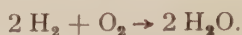
In a recent publication of LEE and UMBREIT (9) experiments are described in which crude sand cultures showed an active consumption of hydrogen as long as oxygen was also present in the gas mixture, in contrast herewith no hydrogen was consumed when oxygen was absent, although carbon dioxide was amply available. On the other hand the authors conclude from their experimental data that carbon dioxide is essential for the initiation of the hydrogen oxidizing process. From the crude cultures several pure cultures were isolated, two of which were subjected to a closer study. For these strains both autotrophic and heterotrophic growth could be proved.

2. OUTLINE OF THE PRESENT INVESTIGATION.

The studies referred to in Chapter 1 — especially those made by KASERER, NIKLEWSKI and RUHLAND — leave no doubt that at least several hydrogen oxidizing bacteria have the ability of autotrophic growth, *i.e.*, that they are able to proliferate in a medium in which carbon dioxide is the only available carbon compound.

Now it has to be acknowledged that the way in which chemo-autotrophic organisms manage to convert carbon dioxide into cell material is still completely wrapped in mystery. As a rule comment is restricted to the remark that the energy derived from the oxidation of the inorganic substrate is amply sufficient to meet the energy requirements involved in the reduction of the carbon dioxide. However, for the better-known autotrophic bacteria, like the sulphur bacteria and the nitrifying bacteria, scarcely any attempt has been made to specify the chemical reactions which lead from carbon dioxide to cell material. Yet in the case of the last-mentioned autotrophs it seems certain that the oxidation of the inorganic substrates proceeds in stages, involving the formation of several intermediate products. For this reason it remains quite conceivable — and it has in fact been suggested — that it is a reaction between one of these intermediates and the carbon dioxide which leads to a primary reduction of this molecule.

In the case of the hydrogen oxidizing bacteria this „intermediate hypothesis”, however, can at once practically ¹⁾ be ruled out, the energy yielding reaction being restricted to the simple equation:



For this reason the hydrogen oxidizing bacteria seem to offer an exceptionally favourable subject for a closer study of the chemistry of chemo-autotrophy.

In the light of this introduction it seems desirable to report here briefly the views regarding this point as laid down in the publications reviewed in Chapter 1.

The earlier investigators like KASERER and NIKLEWSKI were inclined to accept the idea that the primary reaction is one between hydrogen and carbon dioxide leading to the formation of some product of carbohydrate nature and that the oxygen only reacts with this organic substrate in an ordinary respiration process.

Thus for instance KASERER advances the following equations for the metabolic changes involved:



As will be seen KASERER rejects the idea of a direct reaction between hydrogen and oxygen. It must, however, be remarked that his scheme bears a fully hypothetical character, since KASERER does not offer any experimental proof that hydrogen consumption also occurs in the absence of oxygen.

As a result of his extensive investigations RUHLAND has arrived at a very different view which agrees fairly closely with that put forward by LEBEDEFF in 1909. RUHLAND concludes from his experiments that *Bacillus pycnoticus* is indeed able to oxidize hydrogen according to the equation:



As for the carbon dioxide assimilation a plant physiologist like RUHLAND could in 1924 only visualize a reaction between carbon dioxide and water as is realized in the photo-autotrophic green cells, and he thus accepts here as well the following reaction to take place:

¹⁾ The intermediate formation of hydrogen peroxide cannot be excluded, but this does not seem to be helpful for the solution of the problem under discussion.



The assumption, therefore, is made that in some way or other an energy transfer between the reactions I and II occurs, the average efficiency of which was calculated by RUHLAND as to be about 20.5 %.

According to RUHLAND the oxygen evolved in the assimilatory process would be responsible for the fact that in his growing cultures the ratio between the hydrogen and oxygen disappearing from the gas phase was always markedly higher than 2.

The observation that in the experiments in which carbon dioxide was absent the said ratio was distinctly lower than 2 makes, however, RUHLAND conclude that the bacteria in question also bring about a normal respiration process:



The fact that hydrogen oxidizing bacteria are also able to lead a heterotrophic mode of life leaves no doubt that under these conditions such an oxidation does indeed occur. RUHLAND, however, postulates that this normal respiration with the primary assimilation product as a substrate also takes place under conditions of autotrophy, although in this case the simultaneous carbon dioxide assimilation obscures its presence.

In the light of our present knowledge RUHLAND's theory — which has the advantage that it fully corresponds with the current conceptions regarding chemo-autotrophy in other bacterial groups — presents various weak points. It seems quite improbable that the facultatively heterotrophic hydrogen oxidizing bacteria will be able to liberate oxygen from water. In this respect it must be observed that nowadays it is generally accepted that in carbon dioxide assimilation also other hydrogen donors than water may be active, so that there is no necessity whatever to accept RUHLAND's equation II. Moreover the recent developments in our insight into the chemistry of respiration seem to make it superfluous and even illogical to bring about a clear cut separation between the energy yielding reaction I and the normal respiratory process III. Since we have learnt to see the initial step of respiration as a dehydrogenation of the substrate, it appears more probable that the dehydrogenase active in this step will also be responsible for the activation of the atoms in the gaseous hydrogen. In this way the reported inhibitory effect of organic substrates on the hydrogen oxidation can be easily accounted for. The fact that the ability of hydrogen oxidation is not restricted to a small group of specialists, but is so widely distributed

amongst representatives of very different heterotrophic groups speaks also in favour of this view.

Mention may be made of the fact that BURK (10) in a purely theoretical study has also rejected the conclusions drawn by RUHLAND from his experiments, and BURK again advances the view of the older investigators, namely that all the hydrogen consumed is used to reduce carbon dioxide, and all the oxygen consumed is therefore used in respiration. No direct reaction between oxygen and hydrogen would take place.

In the recent publication of LEE and UMBREIT (9) this controversy is not discussed, but it is more or less incidentally stated that the crude sand cultures used in their experiments did not consume hydrogen in the presence of carbon dioxide, if oxygen was absent, a result which seems incompatible with KASERER's scheme.

The foregoing may suffice to warrant the conclusion that the whole problem of the metabolism of hydrogen oxidizing bacteria is still badly in want of further elucidation.

In considering the situation we were struck by the fact that practically all the earlier investigations on hydrogen oxidation had been dealing with experiments of long duration, *i.e.*, with experiments in which the growth of the bacteria was an essential factor. It appeared to us that possibly some progress could be made by studying the behaviour of so-called „resting bacteria” in experiments of short duration, thus avoiding the complications involved in the growth process.

More especially the manometric method seemed well adapted to obtain a better insight into the interrelationship between hydrogen oxidation and normal respiration. In addition this method seemed suitable to give a definite answer to the question whether a reaction between hydrogen and carbon dioxide does occur, if oxygen is absent.

3. ISOLATION OF PURE CULTURES OF HYDROGEN OXIDIZING BACTERIA.

In order to obtain enrichment cultures we proceeded as follows. In an open Petri-dish of 13 cm diameter 50 cc of an inorganic salt solution as used by GROHMANN and containing pro litre distilled water:

1 gram	Na_2CO_3
1 „	NH_4Cl
0.7 „	KH_2PO_4
0.2 „	MgSO_4
0.2 „	NaCl
trace	FeCl_3

was brought. After inoculation with some mud from a ditch the dish was placed at 30° C. under a glass bell, in which the greater part of the air had been replaced by hydrogen and to which 10 % carbon dioxide had been added. In a second experiment the procedure was the same, but this time the medium was inoculated with garden soil. In both experiments there was a marked gas consumption, and after 5 to 7 days a rather thick bacterial film had developed at the surface of the medium. These films were streaked off in the usual way on agar plates having the same mineral composition, which plates were brought in an anaerobic jar containing the same gas mixture as previously used. By replating four different pure cultures were obtained which were labeled Nr. 1 to 4.

Preliminary experiments with these strains either in Eldredge tubes or in an apparatus as devised by SÖHNGEN (11) showed that they all were able to oxidize hydrogen under autotrophic conditions.

All strains proved to be facultative heterotrophs, growth on ordinary peptone agar being quite satisfactory.

For manometric experiments with resting bacteria strain Nr. 2 proved to be the most suitable, since the bacteria when grown autotrophically could readily be collected by centrifugation and from the sediment a homogeneous suspension could be obtained.

A further investigation of strain Nr. 2 showed that it was a Gram-negative motile, non sporeforming rod with lophotrichous cilia. On peptone agar with or without 1 % glucose yellow colonies were formed. Gelatin was not liquefied, fermentative ability was lacking, in litmus milk no acid was formed, the litmus being reduced after some days. Neither indole nor hydrogen sulphide were produced.

These properties are in agreement with the idea that this culture was identical with *Hydrogenomonas flava* of NIKLEWSKI.

4. EXPERIMENTS WITH RESTING BACTERIA.

The first experiments with suspensions of *Hydrogenomonas flava* in the Warburg apparatus were most disappointing. In order to obtain without difficulty sufficiently dense suspensions, the bacterium had been grown under heterotrophic conditions. To our surprise these suspensions when brought in the Warburg apparatus into contact with gas mixtures containing hydrogen and oxygen in various proportions did only show a weak gas consumption identical with that in air, so that evidently no hydrogen had been oxidized.

This negative result induced us to study the behaviour of bacteria grown under strictly autotrophic conditions. With these bacteria indeed

a strong gas consumption occurred in an atmosphere consisting of a mixture of 80 % hydrogen and 20 % oxygen. However, the rate of gas consumption showed a marked decline with time to such a degree that after 30 to 90 minutes the consumption came to a standstill. Apparently the bacteria had died, since addition of an organic respiration substrate like lactate did not restore the gas consuming power.

The fact that NIKLEWSKI reports that for hydrogen oxidation by his *Hydrogenomonas plava* it was necessary to reduce the oxygen content of the gas mixture considerably led to the establishment that a constant rate of gas consumption was only obtained when the amount of oxygen in the gas mixture was reduced to 8 %. This result tends to confirm our identification of strain Nr. 2 with the species in question.

In a first experiment the gas consumption of a bacterial suspension in 0.15 mol phosphate buffer $\text{pH} = 7.2$ in contact with a gas mixture containing 50 % N_2 , 42 % H_2 and 8 % O_2 was compared with the oxygen consumption of the same suspension in air both in the absence and in the presence of an organic respiration substrate. As such an aqueous solution of sodium lactate leading to a final concentration of 1 % in the medium was added from a side-bulb. In all vessels a small quantity of 10 % sodium hydroxide was present in the central compartment in order to absorb the carbon dioxide formed.

If in the first vessel hydrogen would act merely as a respiration substrate, one might expect that the amount of oxygen consumed would be the same as that consumed in the presence of the lactate. Since in the first case the consumption of one volume of oxygen would involve the disappearance of two volumes of hydrogen the total gas consumption should amount to thrice that in the lactate vessel.

For the sake of brevity we will refrain from giving here full particulars regarding this experiment. It may suffice to state that the gas consumption in the "hydrogen vessel" was at least 6 times that in the "lactate vessel" and from 40—50 times as high as the oxygen consumption in the vessel to which no respiration substrate had been added.

This result might still be interpreted satisfactorily by assuming that the capacity of the dehydrogenase system in the bacteria which probably determines the rate of oxygen consumption is larger when molecular hydrogen replaces the organic hydrogen donator.

To test this possibility it was indicated to compare the gas consumption in the simultaneous presence of hydrogen and lactate with that in vessels to which only one of these oxidation substrates was added. This was done in the following experiment.

Four manometric vessels all received 2 cc of a bacterial suspension in 0.15 mol phosphate buffer pH = 7.2, whilst the central vessels all contained sodium hydroxide for the absorption of carbon dioxide formed.

For the rest the conditions were as follows:

Vessel A: the side bulb contained 0.3 cc distilled water

Vessel B: „ „ „ „ 0.3 cc of a 6 % solution of sodium lactate.

In both vessels A and B air was used as gas atmosphere.

Vessel C: as Vessel A

Vessel D: as Vessel B

In both vessels C and D the gas atmosphere consisted of a mixture of 50 % N_2 , 42 % H_2 and 8 % O_2 .

The results of this experiment are represented in Fig. 1.

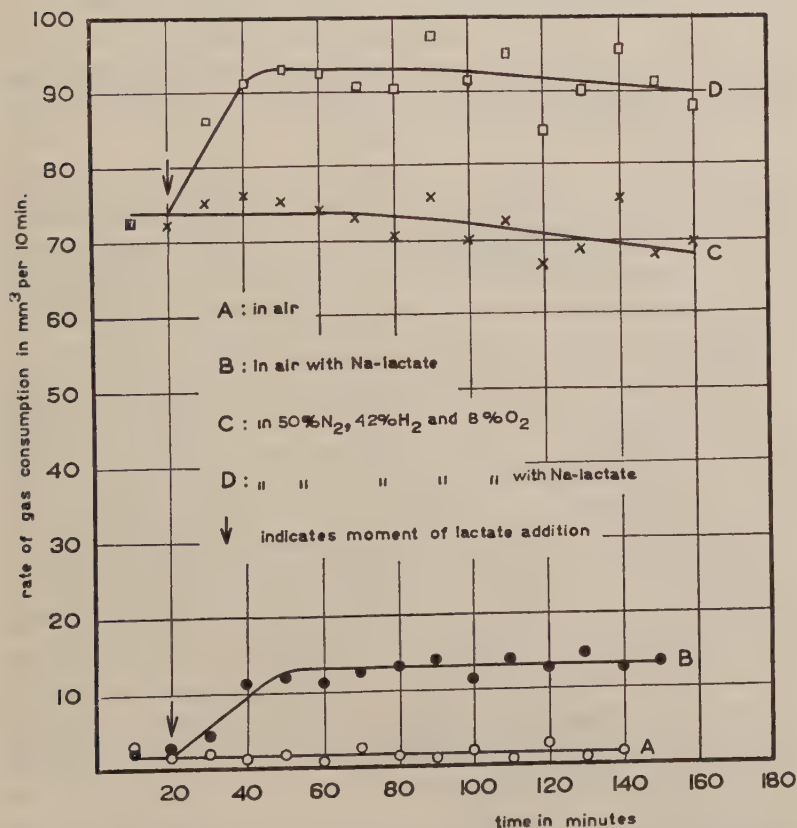


Fig. 1. Gas consumption by *Hydrogemonas flava* in air and in a hydrogen-oxygen mixture with and without Na-lactate.

In this figure the arrows indicate the moment at which the contents of the side bulb were added to the main vessel.

The outcome of the experiment is remarkable from several points of view. In the first place the results are in marked contrast to the current opinion, based mainly on the experiments of NIKLEWSKI and of RUHLAND, that the presence of organic substrates prevents the oxidation of the hydrogen.

To the contrary comparison of the curves C and D shows clearly that the gas consumption in the hydrogen containing atmosphere is also considerably increased by the addition of lactate.

The experiment may be considered a first proof that indeed hydrogen oxidation and lactate oxidation are independent processes as has been postulated by RUHLAND. It seems not well possible to reconcile this observation with the idea that both molecular hydrogen and lactate act as substrates for one and the same respiratory catalytic system. The protective action of organic substrates on the hydrogen oxidation as observed by earlier investigators must apparently be intimately linked up with the growth process.

A second point to which attention is drawn, is, however, that the effect of lactate addition to the hydrogen oxidizing system leads to an increase in rate of gas consumption which is almost twice as large as the rate of oxygen consumption in air. This means that the two gas consuming processes not only do not mutually interfere, but that an unmistakable stimulation of at least one of these processes takes place.

In order to obtain some further insight into what was going on under these conditions we decided to make some experiments in which the amount of lactate was taken so small that in case of oxidation it would be completely consumed within the duration of the experiment. If the effect of lactate addition would depend merely on a stimulation of the hydrogen oxidation one should expect that the gas consumption would remain constant at the high level. If on the other hand the lactate is oxidized simultaneously with the hydrogen the disappearance of the lactate out of the medium should also in the hydrogen containing atmosphere manifest itself in a return to the initial rate of gas consumption.

The results of such an experiment are collected in Fig. 2.

In this experiment 8 Warburg vessels were employed, four of which were filled with air, whilst in four others again a mixture of 50 % N_2 , 42 % H_2 and 8 % O_2 was applied.

In each series the side bulb of one of the vessels contained 0.2 cc distilled water, and the other three the same quantity of a solution of

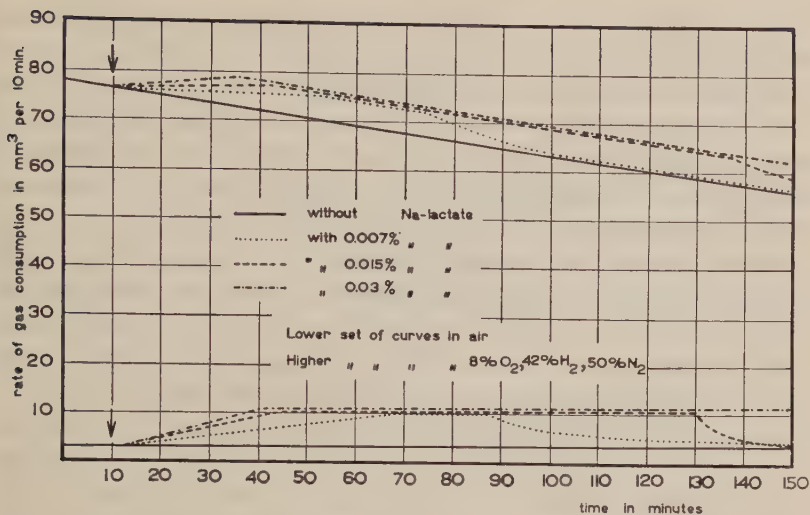


Fig. 2. The effect of the addition of different amounts of Na-lactate on the gas consumption by *Hydrogenomonas flava* in air and in a hydrogen-oxygen mixture.

Na-lactate in concentrations of 0.05 %, 0.1 % and 0.2 % respectively. This meant that after mixing the lactate concentrations in the media became 0.007 %, 0.015 % and 0.03 % respectively.

The outcome of the experiment is self-explanatory. From Fig. 2 it is obvious that the increase of gas consumption by the lactate addition to the medium in the hydrogen containing atmosphere does not only give rise to an increase in gas consumption which is almost equal to the increase in respiration in the air atmosphere, but that this rise in gas consumption extends over the same periods in both cases. This can only mean that also in the hydrogen containing atmosphere the lactate is oxidized at practically the same rate as in the normal respiration experiment. In this case we meet with a more or less ideal superposition of both gas consuming processes, which evidently proceed quite independently. This implies that in *Hydrogenomonas flava* a catalytic system for hydrogen oxidation is present which is quite apart from the catalytic systems involved in the normal respiration process.

We have corroborated these results by determining the so-called „Knallgasquotient”, i.e., the ratio of hydrogen and oxygen consumption, both in the absence and in the presence of lactate. Since the separate determination of the amounts of the two gasses disappearing in the vessels presents great difficulties we proceeded as follows. The vessels were first filled with hydrogen and hereupon an amount of oxygen was

added which was kept so small that it was totally consumed before the end of the experiment.

After several trials the following technique was developed. Oxygen from a bomb was led through an overflow in which a few centimeters of water pressure was maintained. With the aid of a two-way stopcock part of the oxygen at the reduced pressure could be led into a perforated stopper of the side-bulb of the Warburg-cuvette.

Before entering the cuvette the gas had been brought at the correct temperature by passing the gas through a glass spiral which was immersed into the waterbath. A correction for the fact that the capillary of the stopper contained air which was also pressed in could easily be made after the volume of the capillary had been determined.

The amount of oxygen pressed in could be accurately determined by reading the manometer which in these experiments was not filled with the usual Brodie solution, but with a solution of lead perchlorate having double the specific weight, as recommended by KREBS (12). For the preparation of this solution perchloric acid (s.w. 1.5) was neutralized with lead carbonate, after which 0.1 % sodium cholate was added. After filtering off the precipitate the clear liquid was diluted with distilled water till the specific weight at room temperature was 2.068.

The use of this manometer liquid means that the vessel constants had to be doubled. Taking this into account, it was quite feasible to introduce in each Warburg vessel equal volumes of oxygen.

Preliminary experiments showed that at the moment of total consumption of oxygen the gaseous metabolism practically came to a stand. Since the initial volume of the oxygen was known, it was possible to determine the volume of the hydrogen consumed at that moment from the total amount of gas which had disappeared.

The results obtained in two different experiments were as follows.

Ratio hydrogen/oxygen.

	Without lactate	With 1 % lactate
1st Exp.	2.11	1.60
	2.20	1.67
2nd Exp.	2.08	1.71
	2.14	1.61

From these figures too it is evident that addition of lactate does not lead to a mere stimulation of the hydrogen oxidation, i.e., an increased

consumption of both hydrogen and oxygen, but that this addition specifically increases the oxygen consumption, which can only mean that next to the hydrogen oxidation an oxidation of the lactate also takes place.

The second point which we have investigated is the question whether *Hydrogenomonas flava* is able to promote a reaction between hydrogen and carbon dioxide as postulated by KASERER and others. In order to study this question the autotrophically grown bacteria have been suspended in an bicarbonate buffer and the suspension brought into contact with hydrogen. Although we have repeated this experiment several times, in no case any decrease in pressure could be observed. In all cases we have convinced ourselves that the bacteria were in an active state by admitting after some time a small quantity of oxygen. This always led to an immediate gas consumption.

5. DISCUSSION OF RESULTS.

Although this investigation bears only a preliminary character, a few conclusions seem to be warranted.

For a typical hydrogen oxidizing bacterium, in all probability identical with *Hydrogenomonas flava* Niklewski, it has been shown that it is possible to superpose a normal respiration on the process of hydrogen oxidation. This result is contrary to what might be expected on ground of various statements in literature, according to which the addition of organic respiration substrates to cultures of hydrogen oxidizing bacteria practically completely inhibits the hydrogen oxidation. Apparently the explanation of this contradiction must be sought in the fact that the addition of organic substrates to the cultures leads to the generation of bacteria of a purely heterotrophic character, lacking the faculty to bring about hydrogen oxidation. Our experiments make it probable that hydrogen oxidation asks for the presence of a special catalytic system which is quite independent of the catalytic system active in the normal respiration. Obviously the formation of the special hydrogen oxidizing system only takes place when the bacteria are grown under autotrophic conditions, a statement which is borne out by our observation that suspensions of *Hydrogenomonas flava* grown under heterotrophic conditions are unable to bring about any hydrogen oxidation when tested as resting bacteria in the Warburg apparatus.

Yet it should be kept in mind that the faculty to produce the catalytic system in question is only gradually lost. Throughout our investigation we have relied on pure cultures of *Hydrogenomonas flava* grown hetero-

trophically. Nevertheless, it remained possible to transfer these cultures successfully in media suitable for autotrophic development, and it were the crops obtained in these autotrophic cultures which have been used in the manometric experiments. However, at the end of our investigation we have been obliged to witness that the autotrophic cultures no longer succeeded; apparently the faculty to build up the special hydrogen oxidizing apparatus had irreparably gone lost by the continued cultivation of the strain under heterotrophic conditions. We are the more inclined to accept this explanation with a view to similar experiences of earlier investigators, in which connection we refer to the paper of GROHMANN (8).

For the problem of chemo-autotrophic carbon dioxide assimilation our results may be deemed of importance, in so far as it has been shown that this assimilation does not take place in the absence of oxygen. Here-with the theory first advanced by KASERER, supported by NIKLEWSKI and more recently by BURK, according to which the primary reaction is one between carbon dioxide and hydrogen, may be deemed to be definitely refuted. Evidently carbon dioxide assimilation can only proceed with simultaneous consumption of oxygen, and for the moment we must conclude that in analogy with what is known regarding the chemo-autotrophy in other bacterial groups the hydrogen oxidation is in some way or other indispensable for the carbon dioxide assimilation. This means, of course, that the riddle of the mechanism of this assimilation remains unsolved.

S u m m a r y.

Enrichment cultures for hydrogen oxidizing bacteria enabled us to isolate four different species having this faculty, one of which apparently was identical with *Hydrogenomonas flava* Niklewski. This strain proved to be suitable for manometric experiments with resting bacteria.

With suspensions of these bacteria manometric experiments regarding the gaseous metabolism have been performed with the ultimate aim of contributing to the solution of the problem of chemo-autotrophic carbon dioxide assimilation.

It was found that the bacteria only brought about hydrogen oxidation, if they had also been grown under autotrophic conditions; heterotrophically grown bacteria did not oxidize molecular hydrogen, although they were able to respire normally with organic substrates.

In contrast herewith the autotrophically grown bacteria proved to

be able to bring about both types of oxidation. Moreover, on addition of both hydrogen and lactate these substrates proved to be oxidized simultaneously, the rate of each of these oxidations being unimpaired. Under certain conditions the total gas consumption was even unmistakably enhanced. These results seem to warrant the conclusion that hydrogen oxidation asks for a special catalytic system independent of the catalysts active in the normal respiration process. Apparently this special system is only built up when the bacteria are grown under autotrophic conditions, and the faculty to synthesize this system is gradually — and after some time irreparably — lost on cultivation under heterotrophic conditions.

Finally it has been shown that bacteria, which were quite active as far as hydrogen oxidation was concerned, were completely unable to bring about a reaction between hydrogen and carbon dioxide. Herewith the theory regarding the nature of the metabolism of hydrogen oxidizing bacteria already advanced in 1906 by KASERER and still recently defended by BURK has been definitely refuted.

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(From the Laboratory of Hygiene of the University, Utrecht).

ON THE ACTION OF SULFANILAMIDE

VII. A SULFANILAMIDE ACTIVATING SUBSTANCE IN HORSE BLOOD ¹⁾

by

K. C. WINKLER and H. W. JULIUS

(Received July 23, 1942).

WOLFF and JULIUS (2) reported that the bacteriostatic action of sulfanilamide in vitro on streptococci was evident in media containing horse blood, but next to absent in media, containing an equal part of sheep blood. These authors explained the difference by supposing a better growth in horse blood. Better growth — that is more divisions per time-unit — being, in our views at that time, necessarily correlated with increased sulfanilamide action.

A further study of the different activity of sulfanilamide on streptococci cultured in horse and sheep blood, revealed some striking features on which we wish to report here.

The used strain (*Streptococcus haemolyticus* strain ARONSON) and the method by which viable bacteria were counted were the same as in our previous experiments (1). Sterile blood was obtained from several horses and sheep, during several years from the stables of the „Rijks Instituut voor de Volksgezondheid”. If not stated otherwise, the streptococci were grown and investigated in infusion broth with 50% horse blood or sheep blood. The concentration of sulfanilamide generally was 0.015%. In the figures the log. of count is calculated per 28 mg of the medium. To obtain the log. of count per ml log. 35 should be added.

A. AN ACTIVATING SUBSTANCE IN HORSE BLOOD.

Fig. 1 shows the action of sulfanilamide on streptococci, growing in horse and sheep blood. The difference is very striking. This set of curves

¹⁾ Sixth communication: K. C. WINKLER, *Antonie van Leeuwenhoek* 8, 10, 1942.

is typical and has been reproduced in innumerable experiments. No better sulfanilamide action could be obtained in the sheep blood medium

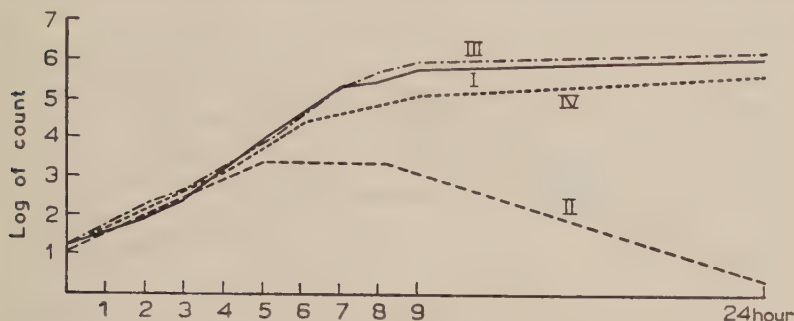


Fig. 1. Streptococci in infusion broth with 50% blood.

I. horse blood. II. idem with 0.015% sulfanilamide.
 III. sheep blood. IV. idem with 0.015% sulfanilamide.

either with higher concentrations of sulfanilamide up to 0.15% or after longer periods. With sulfapyridine the same difference was observed.

The difference was not connected with possible phagocytosis or other influences of blood cells as practically identical curves were obtained with haemolysed blood.

To explain this difference between horse and sheep blood one might suppose:

- a) an inhibiting factor in sheep blood (para-aminobenzoic acid?)
- b) an activator in horse blood
- c) a combination of a) and b):

In infusion broth with a mixture of equal parts of horse and sheep blood the curve for the sulfanilamide containing culture was found practically in the middle between curves II and IV in fig. 1. So this experiment could not decide between the presumed possibilities, but we will retain this fact for further discussion.

To detect a possible inhibition and its whereabouts in sheep blood, horse blood was centrifuged, the supernating serum drained off and the cells resuspended in sheep serum. Fig. 2 shows that sheep serum does not inhibit the sulfanilamide action (curve VI).

By adding washed red cells of a sheep and also haemolysed sheep cells to horse blood it was further ascertained that no inhibiting factor was present in sheep blood.

By adding horse serum to sheep blood or to red cells of a sheep, horse serum was found devoid of activating factors. Red cells of the horse

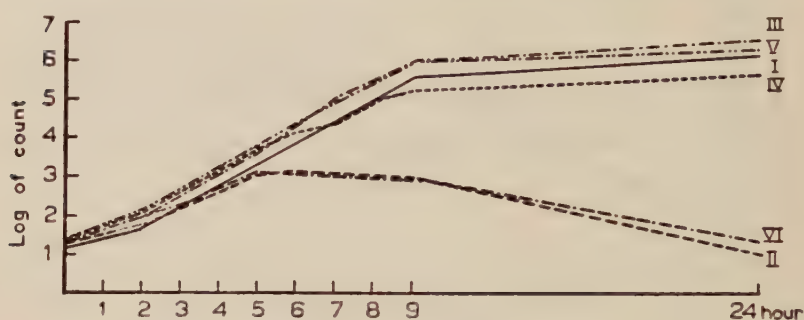


Fig. 2. Streptococci in infusion broth with 50% blood.

- | | |
|-------------------------------|-------------------------------------|
| I. horse blood. | II. idem with 0.015% sulfanilamide. |
| III. sheep blood. | IV. idem with 0.015% sulfanilamide. |
| V. horse cells + sheep serum. | VI. idem with 0.015% sulfanilamide. |

however contained an activating principle, whether the cells were haemolysed or not, as is evident from fig. 3.

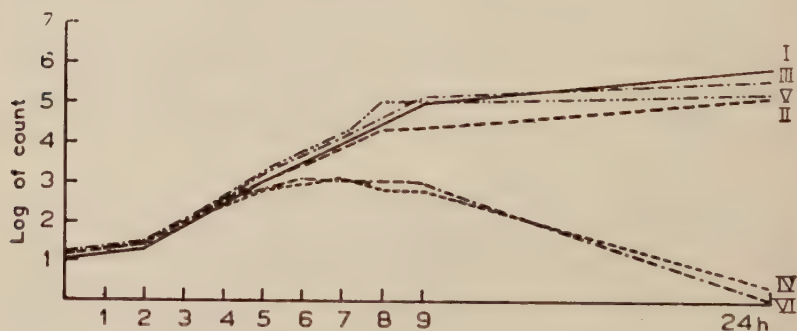


Fig. 3. Streptococci in infusion broth with 50% blood.

- | | |
|--|------------------------------|
| I. sheep blood. | II. idem with sulfanilamide. |
| III. sheep blood + washed horse cells. | IV. idem with sulfanilamide. |
| V. sheep blood + haemolysed horse cells. | VI. idem with sulfanilamide. |

From the control curves it is evident that growth in all these media is equally good. In experiments with haemolysed cells the actual blood concentration is no longer 50% as water had to be added, but here the controls again show that this lowering of the blood concentration does not impair growth.

We may conclude that horse red cells contain a substance or substances which effectively activate the action of sulfanilamide on streptococci in vitro.

Further experiments (Fig. 4) make it evident that the active substance is contained in the red cells, as haemolysed cells are much more activating than a suspension of red cells in saline of the same concentration.

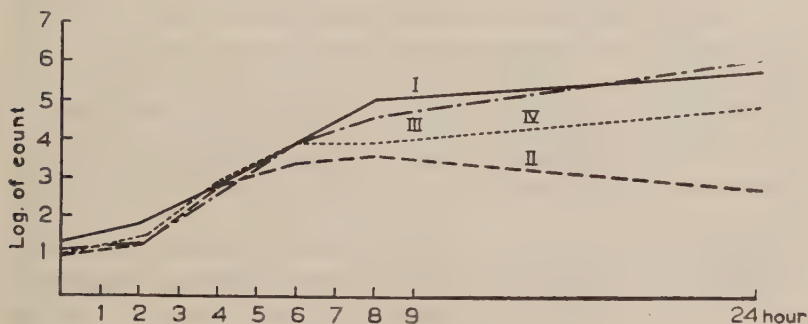


Fig. 4. Streptococci. Medium 1.25 ml sheep blood + 1.25 ml horse red cells 2.5% in saline or distilled water + 2.5 ml infusion broth.

- I. sheep blood + haemolysed horse cells final concentration 0.625%.
- II. idem with sulfanilamide.
- III. sheep blood + horse cells in saline final concentration 0.625%.
- IV. idem with sulfanilamide.

In fig. 4 the control curve with none but sheep blood is omitted, but the obtained curves always were so constant that one might take the curve out of fig. 1, 2 or 3 instead, though the concentration of sheep blood is merely 25% in this case.

From fig. 4 it can be observed that the principle in horse blood is active in great dilution. This was ascertained several times, fig. 5 being an other typical experiment.

It is evident that on the addition of haemolysed cells in a concentration of 2% the medium becomes sterile after 24 hours. With 0.5% haemolysed horse cells the action begins to decrease. It will be clear that not every sample of horse blood was equally active but generally the active dilution was of this order.

Streptococci growing poorly in most media without blood, a medium containing some kind of blood was always used up till now. Still it was

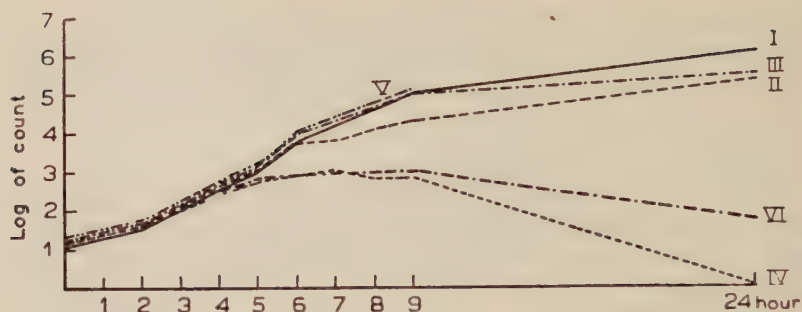


Fig. 5. Streptococci. Medium 1.25 ml sheep blood + 2.5 ml infusion broth + 1.25 ml haemolysed horse cells or infusion broth.

- I. sheep blood. II. idem with sulfanilamide.
 III. sheep blood + haemolysed horse cells final concentration 2%.
 IV. idem with sulfanilamide.
 V. sheep blood + haemolysed horse cells final concentration 0.5%.
 VI. idem with sulfanilamide.

interesting to know whether haemolysed horse cells would also activate sulfanilamide in other media, preferably without the complicating sheep blood. The action of sulfanilamide on streptococci cultured on glucose infusion broth is poor, cultures not becoming sterile at all. By adding 0.5% haemolysed red cells the culture becomes sterile in 36 hours as is shown in fig. 6. The addition of the haemolysate however produces better growth in the control culture without sulfanilamide (curve III fig. 6).

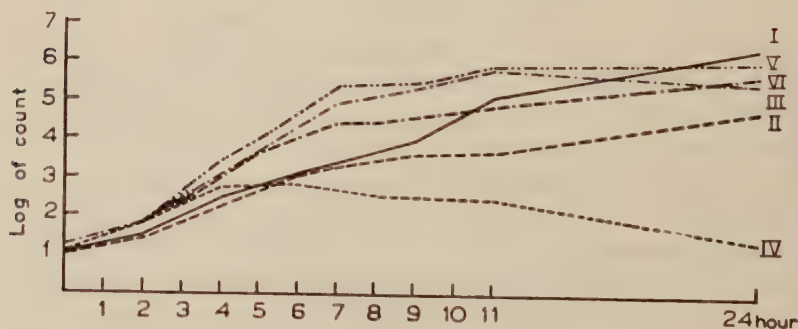


Fig. 6. Streptococci. Glucose infusion broth.

- I. glucose infusion broth. II. idem with sulfanilamide.
 III. glucose infusion broth + haemolysed horse cells 1/100.
 IV. idem with sulfanilamide.
 V. glucose infusion broth + haemolysed sheep cells 1/100.
 VI. idem with sulfanilamide.

To decide whether this increase in growth rate was the cause of a better sulfanilamide action, control cultures were added which contained 0.5% haemolysed sheep cells. This addition also increases the growth rate, but the sulfanilamide action is not enhanced by sheep blood, but of the same order as in the case of glucose infusion broth. Again we may conclude that a very active sulfanilamide activator is present in horse red cells, whilst this action is not due to an increase in the growth rate.

B. WHAT KIND OF SUBSTANCE OR SUBSTANCES ARE INVOLVED?

Small differences in pH or cation content of sheep or horse blood could not possibly explain the described effect. The influence of pH, K^+ or Ca^{++} ions was duly excluded.

Haemolysed red cells, kept at 4° C., retained their activity for about two weeks, whilst washed red cells lose their activity in two days, though whole blood keeps active for two or three weeks. The active principle is not found in the supernate of washed cells. Perhaps autolytical processes play a rôle here, but one has to suppose then, that the autolytical enzymes are destroyed by haemolysis.

By precipitating the stromata of the red cells with HCl and neutralising the supernate, it was shown that the active substance was present in the latter and therefore, in the cell contents and not in the cell membrane or stroma. Stromatine and the cell lipids, practically all of which are contained in the stroma, are thus ruled out.

The haemoglobin solution, containing the active principle, was filterable through a Chamberland filter (L5) without much loss of activity. The active substance was not dialysable through cellophane or ordinary collodium membranes. By adding 1 ml 6% acetic acid to 100 ml 4% collodium in a mixture of alcohol and ether, coarse membranes could be obtained, which let pass the substance, though also part of the haemoglobin passed out of the solution. Adsorption at the collodium membranes was excluded. Experiments on this line are still in progress.

The active substance in the stroma-free haemolysate is always precipitated together with the proteins f.i. by trichloroacetic acid, tannin, alcohol or saturated $(NH_4)_2SO_4$.

At 60° C., as heat coagulation of the protein (haemoglobin) sets in, the substances are destroyed as well.

By dissolving the precipitate with $(NH_4)_2SO_4$ in distilled water and

removing the excess of $(\text{NH}_4)_2\text{SO}_4$ by dialysis, the substance could not be recovered (presumably due to a coarsing of the protein particle).

These facts lead to the supposition that the active principle is a protein or is adsorbed to the protein, which in this case can hardly be other than the haemoglobin. Further experiments are in progress.

C. MECHANISM OF ACTION.

Is the activating substance only necessary to start the sulfanilamide action or has the substance to be present till the end of the process? To answer this question it would be necessary to take the horse cell lysate out of the culture after some hours, in order to observe whether the action of sulfanilamide was continued without activator.

The horse cell lysate cannot possibly be taken out of the culture, but the „taking out” can be realised by diluting the culture a hundred times, this sufficing to render the lysate inactive. By diluting with a medium containing sulfanilamide, it was possible to decide whether the sulfanilamide action continues without the activator. Several controls were necessary; so in the final experiment the culture, containing sulfanilamide and activator was diluted after six hours of growth:

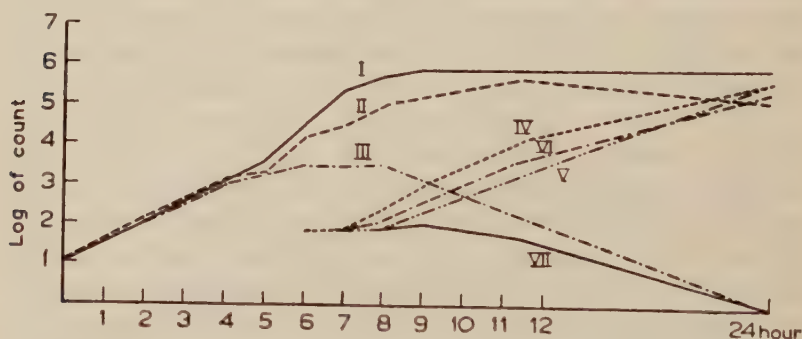


Fig. 7. Streptococci. Infusion broth with 50% sheep blood.

- I. 50% sheep blood infusion broth.
- II. idem with sulfanilamide.
- III. idem + haemolysed horse cells 1/125 with sulfanilamide.
After six hours small samples out of culture III were diluted 100× in:
- IV. 50% sheep blood infusion broth.
- V. idem with sulfanilamide.
- VI. 50% sheep blood infusion broth + haemolysed horse cells 1/125.
- VII. idem with sulfanilamide.

- a) with medium (fig. 7, curve IV).
- b) with medium with sulfanilamide (curve V).
- c) with medium with activator (VI).
- d) with medium with activator + sulfanilamide (VII).

From fig. 7 it is evident, that the sulfanilamide action only continues (curve VII) if sulfanilamide and activating substance are present side by side. With sulfanilamide without activator (V) or with activator without sulfanilamide (VI) growth practically equals the control (IV).

The activating substance is not only starting the sulfanilamide action, but is necessary till the end.

By adding the activator to the cultures after different periods, it was shown that sulfanilamide action only begins when the activator substance is present (fig. 8).

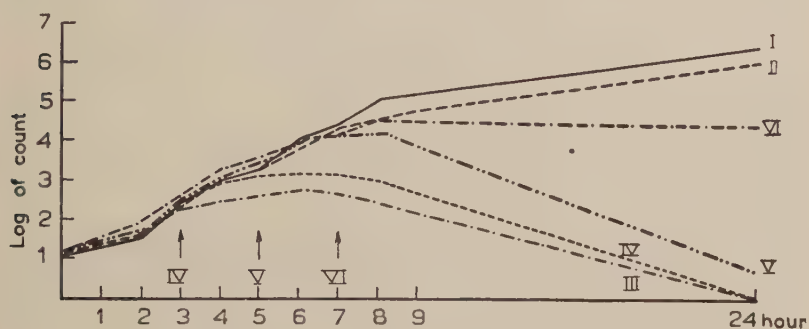


Fig. 8. Streptococci. Infusion broth with 50% sheep blood.

- I. sheep blood.
- II. sheep blood with sulfanilamide.
- III. idem + 2% haemolysed horse cells added immediately.
- IV. idem + 2% haemolysed horse cells added after 3 hours.
- V. idem + 2% haemolysed horse cells added after 5 hours.
- VI. idem + 2% haemolysed horse cells added after 7 hours.

This experiment is a striking counterpart of the experiment from WOLFF and JULIUS (2), where sulfanilamide was added after different times and practically the same set of curves was obtained.

In **this** medium the substance in horse cells is necessary for sulfanilamide action.

D. OTHER MICRO-ORGANISMS.

The sulfanilamide activating influence of haemolysed horse cells

was not limited merely to streptococci. With *Staphylococcus haemolyticus* (isolated from a boil) a marked activation of sulfanilamide was obtained. Sulfanilamide does not act on these staphylococci in glucose infusion broth but after addition of haemolysed horse cells in various concentrations a marked activation was obtained (fig. 9).

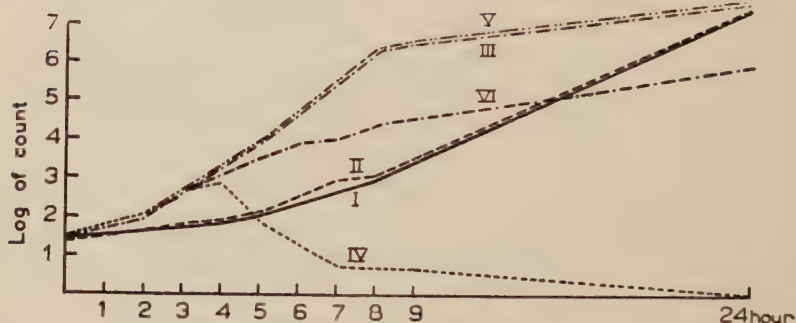


Fig. 9. Staphylococci. Infusion broth with 1% glucose.

- I. glucose broth. II. idem with sulfanilamide.
 III. glucose broth + 2% haemolysed horse cells.
 IV. idem with 0.1% sulfanilamide.
 V. glucose broth + 0.4% haemolysed horse cells.
 VI. idem with 0.1% sulfanilamide.

In a parallel experiment with haemolysed sheep cells no such activation became evident though the growth rate was increased to the same extent as in fig. 9.

With *Bacterium coli* in peptone water an activation, though to a lesser extent, was obtained as well.

E. OTHER KINDS OF BLOOD.

Experiments with blood of rabbits and mice and also with human blood showed that no activating properties were present in these kinds of blood. In media containing these kinds of blood sulfanilamide acted on streptococci such as in sheep blood (compare fig. 1).

Discussion.

The presence of an activating substance in horse blood explains part of the many contradicting statements about the influence of blood on sulfanilamide action. An isolation and identification of the activator might contribute to our knowledge of the mechanism of

sulfanilamide action. Perhaps some therapeutical value might be expected. Experiments *in vivo* were not considered yet.

Awaiting further experimental data the mechanism of the activator action could be explained:

- a) by a possible destruction of an anti-sulfanilamide. The dilution in which the horse lysate is active does not favour this view. Furthermore very small concentrations of para-aminobenzoic acid counteract sulfanilamide even with high concentrations of horse lysate present.
- b) by a damaging of the streptococci by the activator. This explanation does not sound very probable, as the lysate improves growth when sulfanilamide is absent (fig. 6).
- c) by an improvement either of penetration into, or adsorption in the cocci of sulfanilamide. In connection with other experiments, to be described later, this presumption might be possible.

It is still to be pointed out that the experiment with a mixture of horse and sheep blood, mentioned under A seems to indicate that there is, though small, also an inhibiting factor in sheep blood.

As a great many of our previous experiments (2) were made in infusion broth with horse blood one might suggest that the conclusions derived from these experiments, were of doubtful value with regard to the general problems of sulfanilamide action. By special experiments with different media, it was shown however, that the conclusions arrived at in previous publications hold true as well when no horse blood is present. We only wish to recant our previous presumption, that the different action of sulfanilamide in horse and sheep blood should be due to differences in growth rate.

S u m m a r y.

A sulfanilamide activating principle was found to be present in red cells of the horse. This activator substance is active in the rather high dilution of 0.5% haemolysed red cells.

The substance or substances are present in the red cells, not in their cell membranes. They seem to be of a protein nature or adsorbed to the protein (haemoglobin).

In some media no sulfanilamide action is obtained without the activator. In other media sulfanilamide action, though clearly present, is markedly enhanced. So it must be emphasized, that the substance under discussion is an activator and not a „*conditio sine qua non*” for the sulfanilamide action and its characteristics.

The substance is activating sulfanilamide against streptococci, staphylococci and *B. coli*.

The substance is not present in human blood or in the red cells of sheep, rabbits or mice.

References.

1. H. W. JULIUS, *Antonie van Leeuwenhoek* **5**, 28, 1938. – 2. L. K. WOLFF et H. W. JULIUS, *Ann. de l'Inst. Pasteur* **62**, 616, 1939.
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ABSTRACTS

A. F. VAN DER SCHEER, Over mastitis veroorzakende streptococci (On Streptococci causing mastitis). Diss. Wageningen 1941.

In this thesis a detailed study of the biochemical and serological characteristics of the streptococci causing mastitis in cattle is described. The strains required for this study were isolated from cow-milk and uddersecretion, which was furnished to the State serum-institute at Rotterdam by veterinary surgeons.

It is claimed that the various names, used for the strongly haemolytic streptococci of the serological group C, occurring in several infections of our domestic animals, are all systematically incorrect. Moreover several of these names may easily lead to a confusion with *Str. pyogenes* Rosenbach. Therefore the new name of *Streptococcus pyosepticus* is suggested for the haemolytic group C-streptococci.

So the group of streptococci studied in detail will consist of: *Str. agalactiae*, *Str. dysgalactiae*, *Str. uberis*, *Str. pyosepticus* (var. *animalis* and var. *humanus*) and *Str. pyogenes*.

Simplification of methods.

For the biochemical tests the methods described in literature were followed in almost every part. The detection of the haemolytic property was greatly simplified by the use of stab-cultures in blood-agar-slants. The results thus obtained are well in agreement with those arrived at when the original method of BROWN is followed, who inoculated a series of dilutions in blood-agar-plates.

For the serological differentiation of the streptococci, the praecipitin-test of LANCEFIELD was used. Sera were prepared in the usual way. For the preparation of extracts however a strongly simplified method was worked out:

To the sediment centrifugated out of 2 or 3 cc glucose-broth-culture (horse-meat-infusion) two drops of formamide are added and the streptococci are dissolved by cautious boiling over a low flame. After cooling the concentrated extract is diluted to the volume of the original broth-culture with a physiological salt-solution (eventually supplied with phenolred as an indicator). The slight turbidity induced by the mixing is centrifugated off and the clear supernatant is poured into a fresh tube and neutralised.

The precipitin-test is carried out in very small tubes by mixing drops of serum and extract in the ratio of 1 : 2, 1 : 1 or 2 : 1, according to the strength of the sera available. After two hours at room temperature the results are read. The use of higher temperatures (e.g. 37° C. as is often indicated) should be avoided, as in the case of weak sera a positive reaction may frequently fail to occur.

Results.

A full description of the streptococci will be omitted here. Only new data and disputed characteristics will be discussed.

The examination of 312 strains of *Str. agalactiae* showed that the slimy consistence of the colonies of 80 to 90 % of the strains on horse-serum-agar (as described by DIERNHOFFER) is of great importance for a quick recognition of this species. Moreover about 60 % of the strains with non-slimy colonies can be recognised by the colonies „in shape of wound thread” as described by SEELEMANN. Nearly 50 % of the strains induce haemolysis. The area of haemolysis is usually small, but about 10 % of the haemolytic strains produce very large areas. These last results are in contrast with those of other European authors but agree with the data recorded in American publications.

The statistical data collected show that, as might be expected, by far the most of the cases of streptococcal mastitis in cows, (77 %), are caused by *Str. agalactiae*.

The examination of 175 strains of *Str. dysgalactiae* showed that the colonies are never slimy or in „shape of wound thread”. On horse-serum-agar however about 95 % of the strains cause a turbidity in the agar under each colony. After removal of the colonies from the surface of the agar, this phenomenon is very conspicuous. This new characteristic is of great importance for a quick recognition of this species.

Until now it was regarded as an established fact, that neither salicin nor sodium hippurate were attacked by *Str. dysgalactiae*. In this study it was shown however, that some 30 % of the strains show a slow production of acid from salicin and some 20 % cause a weak hydrolysis of sodium hippurate. By means of the precipitin-test and reciprocal absorption-tests it was proved that *Str. dysgalactiae* belongs to the serological group C and that the deviating strains just mentioned should in fact be regarded as *Str. dysgalactiae*.

The statistical data made it apparent, that the number of cases of streptococcal mastitis caused by *Str. dysgalactiae* (14 %) should not be

neglected. Especially during the later half of the year this udder-inflammation is frequent.

83 strains of *Str. uberis* were examined. The surface colonies on horse-serum-agar often show a close resemblance to those of *Str. agalactiae*. Even slimy colonies may occur. A few strains may cause a turbidity in the agar like *Str. dysgalactiae*.

Contrary to the original observations by EDWARDS, it was demonstrated that some 30 % of the strains of *Str. uberis* do not form acid from inulin. Moreover the hydrolysis of sodium hippurate is extremely weak in several cases, so that the results may easily be recorded as negative. By means of the precipitin-test and absorption-tests it is proved that these deviating strains should indeed be regarded as *Str. uberis*. Although SEELEMANN indicates, that for streptococci of this species no group specific serum could be prepared, it has been demonstrated that more than 60 % of the strains contain group specific precipitinogens. Group specific sera, however, are only obtained from strains containing no type-specific precipitinogens. The serological group is not indicated by means of a letter, because it is considered likely that *Str. uberis* will prove to belong to group E. This supposition however cannot be substantiated, because a comparison with American sera was impossible.

The number of cases of mastitis caused by this species is rather small (7 %).

The data obtained from the examination of 68 strains of *Str. pyosepticus* var. *animalis* are in complete agreement with those presented by other authors. Acid is formed from sorbitol but not from trehalose. The precipitin-test with group C serum is always positive.

In cows the mastitis caused by this species is rare (2 %) and occurs nearly exclusively during the earlier part of the year. In other animals, especially in horses, *Str. pyosepticus* occurs more or less frequently in various infections.

Str. pyosepticus var. *humanus* was isolated from cow-milk only once, but not in connection with mastitis. Acid is formed from trehalose but not from sorbitol. Group C serum is precipitated.

Str. pyogenes could not be isolated from cow-milk. Four strains of this species were kindly forwarded by another laboratory, where they were isolated from human material. A group specific serum prepared from one of these strains proved to be different from the group C sera, and is

considered as a group A serum. Biochemical tests cannot serve as means of a definite differentiation of these strains from *Str. pyosepticus* var. *humanus*.

A diagnostic medium.

In the course of the research it appeared, that horse-serum-agar is an excellent medium for the growth and isolation of mastitis streptococci. By the addition of 0.05 to 0.1 % aesculin this agar becomes excellently suited for diagnostical purposes. The hydrolysis of aesculin is easily detected under a quartz lamp. When only non-aesculin-splitting strains are considered, the characteristics for the colonies of *Str. agalactiae* and *Str. dysgalactiae* described above become specific. *Str. uberis* and other streptococci, with colonies showing the same characteristics, all split aesculine. Thus more than 90 % of the strains of *Str. agalactiae* and *Str. dysgalactiae* can be immediately recognised. When it is kept in mind that only 40 to 60 % of the strains of *Str. agalactiae* show haemolysis, it is clear that the new medium has a great advantage over blood-agar which is often recommended for the same purpose. Moreover *Str. dysgalactiae* can be recognised neither in nor on blood-agar.

Discussion.

Str. dysgalactiae, *Str. uberis*, *Str. pyosepticus* var. *humanus*, *Str. pyogenes* and most strains of *Str. agalactiae* form acid from trehalose. Only *Str. pyosepticus* var. *animalis* does not attack this carbohydrate. These results are obtained with a brand carefully examined as to its correct composition, and are in agreement with the great majority of publications on this subject. NOTBOHM and SEELEMANN however recorded deviating results. Deviations of completely the same order were obtained with a brand, which was on the market under the name of „trehalose“, but which upon closer examination proved to be no trehalose at all! Thus it is obvious that every fresh lot of trehalose should be examined before use.

The serological differentiation of the streptococci by means of the precipitin test of LANCEFIELD proved to be of great value. The simplifications worked out in the preparation of the extracts make it possible to examine a great many strains in a short time. Nevertheless the difficulties met with in the preparation of the group specific sera remain a serious draw-back as for its general use. Moreover the results of the serological tests should be judged critically, as rather strong cross-reactions may occur. So if the results of this test are not entirely in

agreement with those of the biochemical tests, which for a thorough investigation always have to be performed as well, it is absolutely necessary to carry out absorption tests.

AUTHOR. ABSTRACT.

F. A. M. J. SMITS VAN WAESBERGHE, Onderzoekingen over microben-amylasen (Investigations of microbial amylases). Diss. Delft 1941.

A concise review of the main chemical investigations bearing on the determination of the configuration of starch is followed by a presentation of the actual state of knowledge of enzymological decomposition of starch.

The aims of the investigations reported on in this thesis consist in: 1° the studying of the occurrence of amylolytic properties in various yeast species, including a more detailed study of the amylase occurring in *Saccharomyces fragilis*; and 2° the gaining of insight in the action of the bacterial amylases which occur nowadays on the market as technical preparations and have been recommended e.g. for the brewers industry and next to this a thorough investigation of the products of hydrolysis of starch induced by the latter amylases.

Primarily the various types of amylases are discussed, respectively the saccharogen-amylase and dextrinogen-amylase and the more recent amylophosphatase and phosphorylase. The action of these amylases on starch and the products of hydrolysis induced by them are treated in detail. Moreover the significance of the results obtained with these amylases is discussed as far as they bear on the configuration of starch.

The investigation as to the occurrence of amylases in 64 yeast species, belonging to widely varying genera, has shown that by far the greater number of these possessed a definite faculty to attack starch more or less intensively. This amylolytic faculty however was distributed quite erratically over the various yeast species and showed important differences in the way in which the starch was attacked. In this connection the author compared the various methods by means of which the decomposition of starch might be ascertained qualitatively. It appeared that the chief methods used: *viz.*, the reaction with iodine in solid and liquid media, the change in the viscosity of a starch sol and experimental comparison of growth rate gave diverging results. It could be proven that some species of *Hansenula* and *Saccharomyces* could, although slowly, ferment in pure cultures part of the starch, with the production of carbon dioxide and alcohol.

Experiments with the maceration juice of *Saccharomyces fragilis* Jörgensen, a yeast which had primarily been shown to be free of maltase, produced results which allowed the conclusion that its amylolytic properties could be wholly attributed to the presence of phosphorylase. Its maceration juice appeared namely to break down the starch quantitatively to glucose. In this connection it could be shown that a phosphorylation of the starch to the CORI-ester preceded the formation of glucose.

The occurrence of amylase in bacteria is discussed in detail, next to the preparations of amylase derived from bacteria such as Superclastase, Rapidase, Biolase N-extra and Biolase hochkonz., which are applied in industry. All these preparations appeared to be free from maltase. Further investigations were carried out on the course of the hydrolysis of starch as induced by such bacterial amylases and more especially by Superclastase, while again the products of hydrolysis were exactly determined. By means of biochemical determinations of sugar and the formation of osazone, the occurrence of glucose and maltose could be ascertained with certainty as products of hydrolysis induced by Superclastase. Moreover a polysaccharide fermentable by *Saccharomyces cerevisiae* could be isolated and identified as a trisaccharide.

The last chapter contains a comprehensive discussion of the results obtained, as far as they bear on the configuration of the starch components.

C. S.

P. B. ROTTIER, Fluorometrische en spectrophotometrische bepaling van lactoflavine in micro-organismen (Dosage fluorométrique et spectrophotométrique de la lactoflavine dans les microbes). Thèse, Delft, 1942. Uitg.-Mij. N.V. v. h. Kemink en Zoon, Utrecht.

Le dosage de la lactoflavine se fait presque exclusivement par trois voies, à savoir: biologiquement, par colorimétrie et par fluorométrie. L'auteur, étant d'avis qu'il est encore à démontrer que le dosage biologique donne la quantité totale de lactoflavine, a seulement étudié les méthodes physico-chimiques mentionnées en collaboration avec d'autres membres du groupe de recherche biophysique à Utrecht.

Par la mesure des spectres d'absorption l'impossibilité de purifier suffisamment les extraits sans pertes sensibles en flavine a été démontrée, de sorte que le dosage par colorimétrie est inexact. Seulement par l'application d'une extrapolation des spectres d'absorption complets

des extraits non-purifiés pour déterminer l'absorption de la lumière de ces impuretés il a été possible d'obtenir des résultats approximativement corrects.

Des résultats plus exacts peuvent être obtenus par la fluorométrie. Pour arriver à ce but il faut d'abord être sûr de mesurer exclusivement la fluorescence de la flavine en éliminant celle des impuretés par l'application d'écrans spéciaux. Puis il faut calculer l'influence diminutive sur l'intensité de la fluorescence de l'absorption dans les extraits de la lumière ultraviolette excitatrice et de la présence de substances éteignant la fluorescence. Un diagramme de calibration a été construit permettant la lecture exacte de la quantité d'une substance fluorescente dans un extrait après la détermination de l'intensité de fluorescence de deux dilutions de cet extrait.

L'extraction de la flavine des microbes s'est seulement montrée quantitative quand elle se fait à chaud avec de l'acide sulfurique à 3% à moins. Quelques dosages ont montré que les microbes sont plus riches en flavine qu'on ne l'a crû auparavant. AUTO-RÉFÉRAT.

L. E. DEN DOOREN DE JONG, De voedingsvoorwaarden der bacteriën in verband met hun pathogeniteit (Nutritive conditions of bacteria in connection with their pathogenicity). Chem. Weekblad **39**, 131, 1942. Cf. also Nederlandsch Tijdschrift voor Geneeskunde **86**, 734, 1942 and Vakblad voor Biologen **23**, **38**, 1942.

The pathogenicity of bacteria is, when seen from the standpoint of the evolutionary doctrine, an acquired property, only developed after the existence of higher plants and animals had become possible and after bacteria had succeeded in conquering the natural resistance of organisms.

According to KNIGHT five evolutionary stages can be noted among bacteria: 1. Autotrophic bacteria. 2. Heterotrophic bacteria. 3. Bacteria requiring amino-acids as N-source. 4. Bacteria which need growth-substances to be added to the organic C- and N-compounds. 5. Bacteria which can develop exclusively in animal organisms.

Among bacteria of group 1 and 2 no real pathogenic forms occur, most of these belonging to group 4. Transitions are found between the different stages. The synthetic ability decreases from 1 to 5.

The pathogenicity of bacteria is caused by their forming endo- or exo-toxines. The question arises why a saprophytic bacterium may have started to develop pathogenic properties.

Comparing pathogenic bacteria with saprophytic bacteria of the same genus it becomes evident that, without exception, the pathogenic bacteria put much more complicated demands to nutrition. With the increasing of pathogenicity the bacteria have lost the ability of synthesising certain substances themselves. On the other hand, cultivation on artificial media may decrease the pathogenic ability and increase the enzymatic potentions (*E. typhosa*, *M. tuberculosis*).

It is now supposed that pathogenic bacteria have developed out of saprophytics because through the stay in rich nutritive surroundings the synthetic ability is decreased. The bacteria are now forming toxic substances to attack living tissue in order to get by these means hold of the necessary nutritive substances.

W. B.

(Aus dem Centraalbureau voor Schimmelcultures, Baarn).

BESCHREIBUNG EINIGER NEUER PILZARTEN AUS DEM CENTRAALBUREAU VOOR SCHIMMELCUL- TURES, BAARN (NEDERLAND)

VII. MITTEILUNG

von

F. H. VAN BEYMA THOE KINGMA

(Eingegangen am 6 October 1942).

***Arachniotus dankaliense* (A. Cast.) nov. comb.**

Im Jahre 1937 erschien von der Hand von Sir ALDO CASTELLANI in Rom eine Veröffentlichung über einen Pilz, der von der behaarten Haut eines Kamels isoliert worden war (1). CASTELLANI beobachtete kurze Ketten von Arthrosporen innerhalb der Haare und beschrieb das Aussehen der Kulturen auf verschiedenen Nährböden. Ausserdem erwähnte er das Vorkommen von Chlamydosporen und von kleinen Zellengruppen, umgeben von einer zarten Haut, die er mit einiger Wahrscheinlichkeit für Asci hielt. Er glaubte dann schliesslich den Pilz in das Genus *Trichophyton* einreihen zu müssen unter dem Namen *Trichophyton dankaliense* nov. spec.

Eine Abimpfung dieses Pilzes wurde dem Centraalbureau im April 1938 von C. W. DODGE übermittelt. Die mikroskopische Betrachtung ergab unzweifelhaft die Anwesenheit von Asci mit reifen Sporen. Besonders auf sterilisierten Kartoffelstücken sowie auf Kartoffelagar entwickelten sich nach kurzer Zeit Fruchtkörper, die sich durch die unvollkommene Ausbildung in der Form von unregelmässigen Myzelknäueln als zur Reihe der *Plectascineae* und zur Familie der *Gymnoascaceae* gehörig erwiesen. Bei den Gattungen dieser Familie besteht die Hülle des Fruchtkörpers aus sterilen Fäden, die ein lockeres Geflecht, die Peridie, bilden, welche die das Innere erfüllenden, unregelmässig gelagerten Asci umschliessen. Bei dem vorliegenden Pilze wird die Peridie nicht von einem Gitter aus verflochtenen Hyphen, sondern von

dünnwandigen, gleichartigen, hell- bis rostbraunen Hyphen gebildet, die in grösserer Zahl rundliche Auftreibungen zeigen. Diese wurden von CASTELLANI als Chlamydosporen bezeichnet. HOTSON in seiner Beschreibung von *Arachniotus trisporus* (2) bildet ebenfalls solche Verdickungen am Myzel ab, die er „Food storage cells“, also „Nahrungsspeicher-Zellen“ nennt. Das 2,5—5 μ dicke Myzel erweitert sich an dieser Stelle bis zu 7—12 μ . In einer Deckglaskultur wollte CASTELLANI auch einige „Spindeln“ gesehen haben, jene mehrkammerigen Gebilde mit doppelt konturierter Wandung, die für gewisse Hautpilze typisch sind; ähnliche Bildungen konnten von uns bei genauer Durchmusterung der Kulturen jedoch nicht aufgefunden werden. Wohl beobachteten wir noch in der Peridie der Fruchtkörper üppig wachsender Kulturen eine Anzahl runde braune, doppelwandige Zellen mit einem Durchmesser von 10—12 μ , welche als eine Art „Hülle“-Zellen betrachtet werden können.

Die rundlichen Asci enthalten 8 Sporen, in reifem Zustande von



Fig. 1. *Arachniotus dankaliense*.

- a. Askenknäuel. Vergr. 245 $\times$.
- b. Asken-Anlagen (Ascogonen). Vergr. 750 $\times$.
- c. Askus. Vergr. 750 $\times$.
- d. Hyphe mit blasenartigen Erweiterungen. Vergr. 750 $\times$.
- e. Aeltere Hülle-Zellen. Vergr. 750 $\times$.
- f. Askosporen. Vergr. 750 $\times$.

rostroter Farbe. Sie haben die Gestalt einer Linse und sind ausserdem von einem auffallend dicken Wulst umgeben. Konidienbildung konnte nicht nachgewiesen werden.

Wenn CASTELLANI schliesslich den von ihm entdeckten Pilz ein *Trichophyton* nennt, dabei jedoch zugleich diese, als vorläufig bezeichnete systematische Stellung anzweifelt, so können wir ihm nur beipflichten, denn nach obenstehenden Ausführungen müssen wir denselben zur Gattung *Arachniotus* bringen. Wir schlagen deshalb vor den Namen dieses von A. CASTELLANI isolierten Pilzes abzuändern in *Arachniotus dankaliense* (A. Cast.) nov. comb

Unter dieser Bezeichnung wird er auch in die Sammlung des C. B. S. aufgenommen werden.

Die Beschreibung des Pilzes gestaltet sich folgendermaassen:

Arachniotus dankaliense (A. Cast.) nov. comb.

Mycelium filzig, anfangs gelblich, später braungelb bis rost- oder orangerot.

Fruchtkörper rundlich, von unregelmässiger Grösse, in Massen im Mycelium eingesprengt als Myzelknäuel, deren aus gleichartigen Hyphen bestehenden Peridien die unregelmässig gelagerten Asci umschliessen.

Asci massenhaft, rundlich, 11—13 μ im Durchmesser, an den ascogenen Fäden auf kurzen Stielen erzeugt, 4, 6 oder 8 Sporen enthaltend,

Ascosporen linsenförmig mit äquatorial verlaufendem Wulst, in reitem Zustande von rostroter Farbe, glatt, (6—8) $\times$ (4,3—5,3) μ .

Hülle-Zellen zahlreich, kugelig, braun, mit doppelter Wandung, 10—12 μ im Durchmesser.

Reinkulturen:

Auf Röhrchen nach 1 Monat:

Auf Kartoffel-Agar: Ein krauswolliges, dunkel gelbbraunes Myzel und verhältnissmässig wenige Fruchtkörper.

Auf Kartoffelstück: Das Stück ganz bewachsen von hellgelbbraunen bis rostbraunen polsterartigen Fruchtkörpermassen, welche ein schwammiges Aussehen haben. In älteren Kulturen nimmt das Stück eine fast schwarze Farbe an. Es strömt einen unangenehmen Geruch aus, etwa wie angefaultes Fleisch.

Hab. Auf der behaarten Haut eines Kamels (A. CASTELLANI).

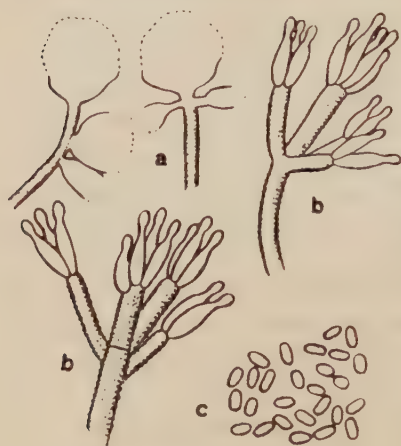
Lateinische Beschreibung:

Ascomatibus rotundis, numerosis, ex hyphis ascos irregulariter diffusos, velut peridium circumdantibus. Ascis rotundis 11—13 μ crassis, 4, 6 vel 8 sporula contigentibus. Sporidiis lentiformibus, margine equatoriali prominente. Si maturis colore rubiginoso, (6—8) $\times$ (4,3—5,3) μ .

Penicillium ingelheimense nov. spec.

Von der Firma C. H. BOEHRINGER Sohn zu Ingelheim a/Rh. erhielten wir im Juli 1941 ein *Penicillium*, das aus der Luft isoliert worden war. Es zeichnet sich durch eine typische gelbbraune Farbe aus, sowie durch seine Neigung zur Bildung von Hyphenbündeln, welche sich

jedoch nicht zu richtigen Koremien zusammenschliessen. Dadurch



Fg. 2. *Penicillium ingelheimense*.

a. Konidienträger. Vergr. 245 $\times$.
b. Konidienträger. Vergr. 750 $\times$.
c. Konidien. Vergr. 750 $\times$.

Penicillium ingelheimense nov. spec.

Rasen älterer Kulturen mit lockerwolliger Konidienschicht und regelmässig über die Oberfläche verteilten polsterartigen Büscheln von Konidienträgern, welche etwas in Farbe variieren von gelbbraun bis rötlich gelbbraun.

Konidienträger 500—800 μ lang und 3—4 μ dick, septiert, rauh, aufsteigend und oft sich zu lockeren Bündeln zusammenschliessend, über der ganzen Länge meist unverzweigt, dagegen am Scheitel mit mehreren, einseitig abgehenden oder sich gegenüber stehenden Seitenästen (Sekundärästen). Sekundäräste mit zahlreichen (etwa 5—10) Aestichen, oft büschelartig vereint, welche je 3—5 Sterigmen tragen.

Sterigmen schlank, spielkegelartig, mit kurzem Halse, 13—16 μ lang und 2,7—3 μ breit, 3—5 an der Zahl.

Konidien wie kurze Stäbchen (Verhältnis Länge zu Breite 2 : 1) mit abgerundeten Enden, seltener daselbst etwas zugespitzt, glatt, hyalin bis leicht gefärbt, in Massen gelbbraun mit rötlichem Stiche, (4—4,7) $\times$ (1,7—2,7) μ (meist 4 $\times$ 2 μ), in langen, gewundenen, parallelen Ketten mit kurzen Disjunkturen, an den Scheiteln der Konidienträger zu falschen Köpfchen von 60—80 μ sich zusammenballend.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 8 Tagen: langsam sich ausbreitende Kolonien, 2,5 cm im Durchmesser; im Zentrum ist die

Farbe durch Konidienbildung 253 D, sonst weiss und flachwollig. Das Mycelium wächst spärlich in dünner Schicht. Rand flach, dünn, unmerkbar in den farblosen, 4—5 mm breiten Saum übergehend. Schwacher Kampfergeruch. Unterseite 278 A—0296. (3).

Auf Röhrenchen nach 8 Tagen:

Auf Bierwürze-Agar: Stark puderig durch zahlreiche aufstehende Konidienträger mit Sporenköpfchen, Farbe zwischen 287 und 288.

Auf Kirsch-Agar: Stark puderig, unten im Röhrenchen 286, oben mit Farbe zwischen 262 oder 287 und 288.

Auf Möhre: Das Stück ganz bewachsen, z. Tl. mit filzig-wolligem Myzel, 278 C—D, z. Tl. mit Konidien, Farbe zwischen 287 und 288. Das Stück nicht gefärbt.

Auf Kartoffel: Das Stück ganz bewachsen, meist mit filzigem, weissem Myzel und wenig Konidien. Das Stück nicht gefärbt.

Auf Raulin-Agar: Eine puderige Konidienschicht, 286—287 oder etwas dunkler. Kein Luftmyzel. Agar farblos.

Auf Reis: 2 cm tief gewachsen, Farbe 287—288. Der Reis nicht gefärbt.

Auf Haterflocken-Agar: Eine dünne Konidienschicht, 287 oder etwas dunkler. Wenig Luftmyzel.

Hab. Aus der Luft isoliert (BOEHRINGER, Ingelheim a/Rh.).

Lateinische Beschreibung:

Conidiophoris longis 500—800 μ , crassis 3—4 μ , asperis, saepe fasciculatis, in apice 5—10 ramis praeditis, omnibus 3—5 sterigmata ferentibus. Sterigmatibus 13—16 μ longis, 2,7—3 μ largis, collo brevi. Conidiis oblongis, si in massa flavobrunneis, (4—4,7) $\times$ 2,7 μ , longis catenis collocatis vel capitulis 60—80 μ .

Phoma suecica nov. spec.

Im Januar 1910 erhielten wir von E. RENNERFELT aus dem Botanischen Institut zu Göteborg einen als *Phoma* A bezeichneten Pilz, der aus feuchtem Holzschliff isoliert worden war, zusammen mit *Phoma lignicola* Rennerfelt. Der Pilz konnte mit keiner der uns bekannten Arten identifiziert werden, wobei der Umstand, dass eine Wirtspflanze nicht angegeben werden konnte die Bestimmung erschwerte. Der Pilz muss jedoch in Schweden heimisch sein, denn von Munksunds Aktiebolag in Munksund erhielten wir unter No. 29 einen aus der Luft isolierten Pilz, der, wie sich später ergab, mit dem Holzschliff-*Phoma* völlig

übereinstimmte. RENNERFELT veröffentlichte über dieses *Phoma* A und zwei weitere *Phoma*-Arten eine Arbeit, worin er über das Verhalten dieser Pilze Quecksilberverbindungen gegenüber ausführlich berichtete (4). Da der Pilz für das Studium des Holzschliffes von Interesse sein dürfte, haben wir denselben im Einvernehmen mit RENNERFELT neu beschrieben.



Fig. 3. *Phoma suecica*.

- a. Fruchtgehäuse. Vergr. 80 ×.
b. Gehäusewand. Vergr. 750 ×.
c. Sporenträger. Vergr. 750 ×.
d. Sporen. Vergr. 750 ×.

Phoma suecica nov. spec.

Fruchtgehäuse herdenweise, stumpfkegelförmig oder niedergedrückt-kugelig, ziemlich klein mit kurzer Mündungspapille, schwarz, glatt

mit getafelter Wandung, glänzend, 160—200 μ im Durchmesser. **Sporen** kugelig bis kurz-elliptisch, ohne Oeltröpfchen, aber mit stark lichtbrechendem Zellinhalt, $(2,7-3) \times (2,3-2,7) \mu$, meist $2,7 \times 2,3 \mu$ gross, in weissen Ballen hervorquellend, welche im Alter nachdunkeln. **Sporenträger** zitzenförmig bis kugelig, hyalin, 5—7 μ breit.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 10 Tagen entsteht eine 6 cm grosse Kolonie aus zahlreichen schwarzen, in breiten Zonen wachsenden Fruchtgehäusen, eingesenkt in einem gleichfarbenen Stroma mit violetter Stiche, alles überwachsen von einem lockeren, braunen Myzel. Um die Kolonie herum ein 3—4 mm breiter farbloser Saum. Die Unterseite ist im Zentrum schwarz, nach dem Rande hin mehr braunschwarz. Auf Röhrchen nach 10 Tagen:

Auf Maismehl-Agar: Ein dichtgeschlossenes violettschwarzes Stroma mit Fruchtgehäusen von derselben Farbe und weisslichem Sporenschleim. Luftmyzel fehlt fast ganz. Die Rückseite zeigt nur einen dunkelbraunen Rand, doch ist sonst farblos.

Auf Kartoffel-Agar: ein dichtes, schwarzes Stroma mit zahlreichen Fruchtgehäusen und weisslichem bis rötlichweisssem Sporenschleim. Luftmyzel fehlt.

Auf Haferflocken-Agar: Ein braunschwarzes Stroma mit zahlreichen Fruchtgehäusen. Die Rückseite ist etwas schwärzlich.

Auf Lupinenstengel: Beginnende Bildung von Fruchthäusern. Nach 25 Tagen hat sich der Stengel schwarz verfärbt und zeigt zahlreiche schwarzen Fruchthäuser mit weissen Sporentröpfchen (Unterschied mit *Phoma lignicola*, wo der Stengel in derselben Zeit farblos bleibt, mit zum Teil noch farblosen Fruchthäusern und fleischrötlichen Sporentröpfchen).

Hab. Isoliert von Holzschliff in Göteborg (E. RENNERFELT). Aus der Luft in Munksund (Schweden).

Lateinische Beschreibung:

Pycnidiis numerosis, in massis, obtuse conicis, satis parvis, brevi ostiolo, nigris, levibus, $160\text{--}200\ \mu$ crassis. Sporulis globosis vel breviter ellipsoideis, aguttulatis, cum protoplasmate lucem frangente $(2,7\text{--}3) \times (2,3\text{--}2,7)\ \mu$, in sporuliphoris papillaeformibus.

Margarinomyces decumbens nov. spec.

Diesen Pilz erhielt das Centraalbureau von POLAK & SCHWARZ's Essence-Fabriken in Hilversum (Niederl.), isoliert von einer Erdbeere.

Auf Agar-Nährböden bildet er submerse Häute, welche von einem weissen Flaum überwachsen sind. Anfangs zeigt die Pilzhaut eine blasse Fleischfarbe, welche bald gelbe oder olivbraune Töne annimmt, bis schliesslich die ganze Pilzdecke aus einer olivbraunen, glatten Haut besteht, worauf nur stellenweise etwas weisses Luftmyzel wächst. Die Konidien werden in grossen Massen von den wenig differenzierten Trägern abgeschnürt. Diese Träger gehen in gewissen Abständen von den fertilen Hyphen ab und enthalten zahlreiche Tröpfchen. Auch das Myzelium, das mit



Fig. 4. *Margarinomyces decumbens*.

- a. Konidienträger. Vergr. $750\times$.
- b. Myzel mit Auftreibungen. Vergr. $750\times$.
- c. Konidien. Vergr. $750\times$.

dem Alter eine braune Farbe annimmt, ist von zahlreichen Tröpfchen erfüllt und bildet leicht Hyphenbündel, welche sich von der Kulturoberfläche erheben, deren Länge jedoch 1 mm nicht übersteigt. Der Pilz steht dem *Margarinomyces Hoffmannii* (5) nahe, von der er sich durch grössere Konidien und rascheres Wachstum unterscheidet.

Die Beschreibung des Pilzes gestaltet sich wie folgt:

Margarinomyces decumbens nov. spec.

Decke filzig-häutig, anfangs glatt, blass fleischfarben, später dunkler werdend, runzelig und olivbraun.

Konidienträger wenig differenziert, nicht massenhaft vom Myzel abgehend, länglich, am Scheitel etwas eingeschnürt, oft im Innern kleine Tröpfchen enthaltend, von sehr verschiedener Länge, (12—60) $\times$ (3—3,3) μ , die Konidien einzeln abschnürend.

Konidien massenhaft, kurz elliptisch, hyalin bis leicht gefärbt, mit 2 Öltröpfchen, (4—6,7) $\times$ (2,3—3,7) μ , meist (4,3—6,0) $\times$ (3—3,3) μ . *Chlamydosporen* wurden nicht beobachtet.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 20 Tagen: Die Schale ganz bewachsen, die Kolonie bestehend aus einer submersen Haut, olivbraun bis braungelb, etwa 187—188, überwachsen von einem lockeren, weissen, filzigen Flaum in Zonen. Rand flach, häutig, 1 cm breit. Schwacher Faulgeruch. Unterseite intensiv gefärbt, etwa 182—192.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: eine dicke, faltige Haut, meist farblos, stellenweise jedoch gelb, etwa 237, von einem kurzen, filzigen, weissen Flaum überwachsen.

Auf Bierwürze-Salep-Agar: wie vorige, nur zeigen die farbigen Stellen diesmal eine mehr orangegelbe Farbe, etwa 182. Um das Impfstück herum hat sich ein schwarzes Stroma entwickelt.

Auf Haferflocken- und Maismehl-Agar: es entstehen farblose, submerse Häute.

Auf Kartoffelstück: das Stück ist von einer glatten, feuchten Haut überzogen, welche teilweise eine intensiv orangegelbe Farbe angenommen hat, etwa 176.

Hab. Isoliert von einer Erdbeere (Fa POLAK & SCHWARZ, Hilversum).

Lateinische Beschreibung:

Conidiophoris e mycelio exeuntibus, velut ramula simplicia, parum declarata, in apice paulum contractis, intus parvis guttulis. Conidiophoris longitudine valde differentibus, (12—60) $\times$ (3—3,3) μ . Conidis numerosis, breviter ellipsoideis, hyalinis vel leviter coloratis duobus olei guttulis, (4—9,3) $\times$ (2—3,3) μ .

***Phialophora atra* nov. spec.**

Diesen Pilz erhielt das Centraalbureau von Herrn S. A. DE LANGE in Amsterdam, der denselben im Anatomischen Laboratorium aus kranken mexikanischen Molchen (Axolotl) isolierte. Herr DE LANGE hatte die Freundlichkeit uns über den Verlauf der Krankheit nähere Angaben zu machen. Demnach kündigt die Krankheit sich an durch Nahrungsverweigerung. Wenige Tage später entstehen am ganzen Körper oft missfarbige graue Flecke, mit deren Erscheinung eine Schwächung der Tiere einher geht. Diese Schwächung scheint die Vorbedingung für den Befall durch Pilze zu sein, wobei besonders die Kiemen schwer heimgesucht werden. Die Schleimhäute schwellen derartig an, dass die Tiere schliesslich durch Atemnot eingehen. Obwohl die meisten *Phialophora*-Arten zu den harmloseren Pilzen zu rechnen sind, die z.B. mit Vorliebe auf Holzschliff und dergl. vorkommen, gibt es doch einige Vertreter dieser Gattung, die parasitisch auftreten können. Bekannt ist in dieser Hinsicht *Phialophora verrucosa* Medlar (6) und *Phialophora macrospora* Moore et Almeida, die beim Menschen eine Chromomycosis verursachen (7), und *Phialophora heteroderae* (Jacz.) van Beyma, die imstande ist, Cysten von *Heterodera Schachtii* zu befallen und zu töten (8, 9). Letzgenannter Pilz wurde wiederholt aus dem Wasser eines Schwimmbades in Rotterdam isoliert. Es ist demnach nicht ausgeschlossen, dass auch der Axolotl-Pilz parasitäre Eigenschaften diesen Tieren gegenüber zeigen kann.

Wir haben diesen Pilz, den wir nicht bei einer der bekannten Arten unterbringen konnten, seiner dunklen Farbe wegen *Phialophora atra* genannt. Von den *Phialophora*-Arten, die wie unser Pilz 4–5 μ grosse Konidien mit zwei Oeltröpfchen bilden, wie z.B. *Ph. aurantiaca* van Beyma, *Ph. heteroderae* (Jacz.) van Beyma und *Ph. lutea-olivacea* van Beyma ist er durch seine dunkle submerse Haut auf Bierwürze-Agar leicht zu unterscheiden.



Fig. 5. *Phialophora atra*.
Phialiden und Konidien. Vergr.
750 $\times$.

Phialophora atra nov. spec.

Myzel unter dem Mikroskop anfangs leicht braun gefärbt, in alten Kulturen dunkler, mit zahlreichen kleinen Oeltröpfchen im Innern, meist etwa $2,7-3,3\ \mu$ breit, verhältnissmässig zart, auf Agar-Nährböden wenig hervortretend.

Phialiden leicht braun gefärbt, von kurzer, gedrungener, mehr oder weniger flaschenförmiger Gestalt, sowohl einzeln wie in kleinen Büscheln beisammen stehend, $8-12\ \mu$ lang, an der breitesten Stelle $3,3\ \mu$ dick, oft kleine Oeltröpfchen enthaltend.

Konidien massenhaft, von den Phialiden einzeln erzeugt, jedoch in feuchter Umgebung zu kleinen Köpfchen zusammenklebend, von sehr verschiedener Grösse, leicht braun gefärbt, mit 2 Oeltropfen versehen, $(4-9,3) \times (2-3,3)\ \mu$, meist $(4,3-5,3) \times (2,3-2,7)\ \mu$.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 20 Tagen: langsam wachsend, Kolonie $4,5\ \text{cm}$ im Durchmesser, bestehend aus einer submersen, feuchten, schwarzen Haut aus feinen, dem Agar flach anliegenden, strahlenförmig vom Impfstück ausgehenden Hyphen, nach dem wie ausgefranst aussehenden Rande in einen $3\ \text{mm}$ breiten farblosen Saum übergehend. Am Rande stellenweise etwas Luftmyzel. Kaum Geruch. Unterseite braunschwarz.

Auf Röhrchen nach 14 Tagen:

Auf Haferflocken-, Maismehl- und Kartoffel-Agar bildet der Pilz schwarze, glatte Häute ohne Luftmyzel. Der Agar wird nicht gefärbt.

Auf Kirsch-Agar: Es entsteht eine schwarze Haut, von dunkelgrauem locker-wolligem Luftmyzel spärlich überwachsen.

Auf Kartoffelstück: Auf das Stück eine glatte, schwarze Haut, von filzig-wolligem, dunkelgrauem Myzel überwachsen.

Hab. Isoliert von den Kiemen eines Axolotl in Amsterdam (S. A. DE LANGE).

Lateinische Beschreibung:

Phialidibus subbruneis, forma compacta, solis vel parvis acervis conjunctis, $8-12\ \mu$ longis, loco crassissimo largis $3,3\ \mu$, saepe intus parvis guttulis. Conidiis numerosis, pituita obductis in parva capitula, subbruneis, duobus olei guttulis, $(4-9,3) \times (2-3,3)\ \mu$.

Phialophora cyclaminis nov. spec.

Diesen Pilz erhielten wir von Dr J. W. M. Roodenburg in Aalsmeer, der denselben von dem Blatte eines weissblühenden Alpenveilchens isolierte. Da er während eines Infektionsversuches zufällig aufgefunden wurde, ist über sein Verhalten der Pflanze gegenüber vorläufig nichts weiter bekannt.

Er zeigt eine gewisse Aehnlichkeit mit *Phialophora brunnescens* (Dav.) Con. (10), weicht jedoch von demselben ab durch die viel kleineren Sporen. Der Pilz wächst auf Agar-Nährböden verhältnissmässig langsam unter Bildung flachfilziger Kolonien von grüngrauer oder grauschwarzer Farbe, welche durch ihren gekerbten, manchmal tief eingeschnittenen Rand auffallen. Die Bildung der Sporen ist im allgemeinen wenig üppig. Auf Bierwürze-Agar werden nur wenige Phialiden erzeugt, besser gedeiht der Pilz auf Kirsch-, Maismehl- oder Haferflocken-Agar. Hier können bei üppiger Entwicklung und in feuchter Umgebung die Konidien zu kleinen Köpfchen verkleben, wodurch der Anschein eines *Cephalosporium* erweckt wird. Das Myzel ist hellgrün gefärbt, meist $4\ \mu$ dick, mit zahlreichen Querwänden und ohne Oeltröpfchen.

Die Beschreibung des Pilzes lautet wie folgt:

Phialophora cyclaminis nov. spec.

Myzel unter dem Mikroskop hellgrün gefärbt, kräftig, etwa $4\ \mu$ dick, ohne Oeltröpfchen und ohne Neigung zur Bildung von Strängen.

Phialiden an den fertilen Fäden nicht massenhaft, meist einzeln stehend, länglich, $16\text{--}20\ \mu$ lang, in der unteren Hälfte mit einer geringen Auftreibung, $3\text{--}4\ \mu$ dick, nach der Spitze zu verjüngt, mit deutlichem „Schnabel“.

Konidien zahlreich, hyalin, rundlich, sehr klein, $1,7\text{--}2,3\ \mu$ im Durchmesser, meist $2\ \mu$.

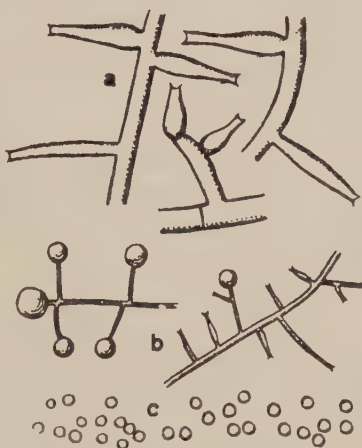


Fig. 6. *Phialophora cyclaminis*.

a. Phialiden. Vergr. $750\times$.

b. Phialiden mit Sporenköpfchen. Vergr. $245\times$.

c. Konidien. Vergr. $750\times$.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 28 Tagen: Langsam sich ausbreitende Kolonie, Schale noch nicht ganz bewachsen. Kolonie bestehend aus einer grüngrauen filzig-wolligen Myzeldecke mit nach dem Rande hin sich überlagernden Zonen und mit tief eingeschnittenem oder gekerbtem Rande. Kein Geruch. Unterseite braunschwarz.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: eine filzig-wollige, grauschwarze Decke mit wenigen Phialiden. Rückseite am Rande braunschwarz.

Auf Kirsch-Agar: geringere Entwicklung wie vorige. Farbe mehr grüngrau, üppige Entwicklung von Phialiden. Rückseite am Rande schwärzlich.

Auf Haferflocken-, Maismehl- oder Kartoffel-Agar: eine filzig-wollige Decke von grauschwarzer Färbung. Rückseite am Rande schwärzlich.

Hab. Isoliert von Cyclamenblatt in Aalsmeer (Niederl.) (ROODENBURG).

Lateinische Beschreibung:

Phyalidibus non numerosis, plerumque solis, oblonge ampullaceis, 16—20 μ longis, crassissimo loco 3—4 μ crassis. Conidiis numerosis, hyalinis, rotundis, minimis, 1,7—2,3 μ .

Tritirachium cinnamomeum nov. spec.

Die Gattung *Spirotrichum* wurde 1939 von SAITO (Japan) aufgestellt. In einer früheren Mitteilung, September 1940 (11) hoben wir hervor, dass eine lateinische Beschreibung dieser Gattung in der Veröffentlichung SAITO's, die ausschliesslich in japanischer Sprache gehalten war, fehlte und demnach den wenigsten Mycologen zugänglich. Anlässlich der Beschreibung einer neuen Art, *Spirotrichum musae*, haben wir dann eine solche Diagnose hinzugefügt. Im Januar-Februar-Heft von *Mycologia* 1940 hat nun D. P. LIMBER (12) unter der Aufschrift: „A new Form Genus of the *Moniliaceae*“ eine neue Gattung, *Tritirachium* aufgestellt und zwei Arten dieser Gattung beschrieben, die offenbar, wie auch die Abbildungen deutlich zeigen, mit denen von *Spirotrichum* sehr nahe verwandt sind. Die fast gleichzeitige Veröffentlichung dieser zu einer und derselben Gattung gehörigen Pilze lässt die Frage nach der Priorität aufkommen und damit nach der richtigen

Benennung auf Grund der bestehenden international vereinbarten Richtlinien. Da nun im Schrifttum eine lateinische Beschreibung von *Spirotrichum* seitens SAITO nicht aufzufinden war, muss dieser Name vor demjenigen des amerikanischen Forschers zurücktreten, sodass die *Spirotrichum*-Arten zur Gattung *Tritirachium* gebracht werden müssen. Demnach soll *Spirotrichum purpureum* Saito jetzt *Tritirachium purpureum* (Saito) van Beyma und *Spirotrichum musae* van Beyma jetzt *Tritirachium musae* van Beyma genannt werden. Hierbei schliesst sich nun eine fünfte Art an, deren Beschreibung unten folgt. Der uns hier beschäftigende Pilz wurde von P. BELS isoliert von Fliegen aus den Kreidehöhlen bei Maastricht (Niederlande) und nach seinen mikroskopischen Merkmalen als zur Gattung *Tritirachium* gehörig erkannt. Da der Pilz mit keiner der nunmehr bekannten Arten übereinstimmt, wurde er als neue Art beschrieben.

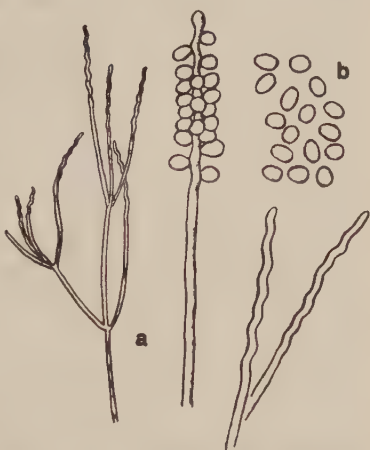


Fig. 7. *Tritirachium cinnamomeum*.

a. Konidienträger. Vergr. 245 $\times$.

b. Träger und Konidien. Vergr. 750 $\times$

Tritirachium cinnamomeum nov. spec.

Rasen verschieden je nachdem die Kultur Myzelien bildet oder haupt sächlich Konidien. Im ersteren Falle entstehen hochwollige, wulstige, weisse Myzelmassen fast ohne Konidienbildung. Im zweiten Falle erinnert das Wachstum an *Tritirachium musae* und *Tr. purpureum*, indem kleine, schwach gelbliche Kolonien gebildet werden, welche, obwohl stark puderig, jedoch wenig Neigung besitzen sich über den Nährboden auszubreiten.

Konidienträger aufrecht, septiert, 1,3—1,5 μ dick, am Ende mit mehreren quirlständigen oder opponiert stehenden Aesten versehen.

Aeste erster Ordnung wenige an der Zahl, meist 3—4, gewöhnlich sehr lang, 100 μ und darüber. Aeste zweiter Ordnung in Wirteln, meist 4 an der Zahl, mit hin und hergewundenen (zackigen) Endauszweigungen, 60—80 μ lang.

Konidien meist gleich gross, regelmässig elliptisch, glatt, hyalin, in Massen hellgelbbraun, (3,3—4,7) $\times$ (2,7—3,3) μ , meist (3,7—4) $\times$ (2,7—3) μ , an den Zacken abgeschnürt, fast ährig stehend.

Vergleicht man die Konidiengrösse dieser Pilzes mit derjenigen der übrigen Arten, so ergibt sich, nach ansteigenden Dimensionen der Konidien geordnet, die Reihenfolge:

Tr. album (1,6—2,5) $\times$ (1,7—3,4) μ ; *Tr. musae* (2,7—3,3) $\times$ (1,7—2) μ ;

Tr. purpureum (2,3—2,7) $\times$ (1,7—2,3) μ ; *Tr. dependens* (2,1—3,3) $\times$ (2,8—3,6) μ ; *Tr. cinnamomeum* (3,3—4,7) $\times$ (2,7—3,3) μ .

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 14 Tagen: Kolonien sich nur langsam ausbreitend, hochwollig, an den Berührungsstellen oft verdickte Ränder bildend, weiss, im Zentrum anfangend sich braungelblich zu verfärben. Rand scharf. Unterseite meist hohl, 161—166.

Auf Röhrchen nach 1 Monat:

Auf Haferflocken- und Maismehl-Agar ausgezeichnete Konidienbildung, wodurch die Kulturen eine hellgelbbraune Farbe angenommen haben. Zwischen den Konidienmassen zahlreiche kleine, farblose Wassertröpfchen.

Auf Kirsch-Agar meist dickwollige Myzelmassen, mit starker Konidienbildung an der Impfstelle.

Auf Kartoffel-Agar stark begrenztes Wachstum, es entstehen kleine flachpuderige, hellgelbbraune Kolonien.

Auf Möhre zahlreiche kleine, puderige, hellgelbbraune, stecknadelkopfgrosse Kolonien, welche kaum mit einander verwachsen.

Hab. Isoliert von Fliegen aus einer Kreidehöhle bei Maastricht (P. BELS).

Lateinische Beschreibung:

Conidiophoris erectis 1,3—1,5 μ crassis, in apice corona praeditis plerumque 3 ramorum primae ordinis, longorum 100 μ vel longiorum, quorum unusquisque 3—4 ramos secundae ordinis gerere potest 60—80 μ longos serratim curvatos in apice. Conidiis equaliter ellipsoideis, levibus, hyalinis, in massa subglavobrunneis, (3,3—4,7) $\times$ (2,7—3,3) μ .

Tritirachium roseum nov. spec.

Aus dem Bakteriologischen Institut der Preussischen Versuchs- und Forschungsanstalt für Milchwirtschaft in Kiel erhielt das Centraalbureau u.a. eine als *Beauveria* spec. bezeichnete Kultur, welche sich bei näherem Studium als ein *Tritirachium* herausstellte.

Ueber die Herkunft des Pilzes wurde angegeben: „Stamm 1316 der HENNEBERG'schen Sammlung. Herkunft nicht mehr mit Sicherheit zu ermitteln, offenbar saprophytisch, wahrscheinlich aus Einstreu in einem Kuhstall isoliert“.

In der Tat ist bei der Gattung *Beauveria* der Hals der Sterigmen oft hin und her gebogen und es werden auch die Konidien an den Zacken abgeschnürt, allein die Verzweigung der Sterigmen ist nicht quirlständig, wie es bei *Tritirachium* der Fall ist, sondern sie stehen hier in dichten, radiär abspreizenden Rosetten beisammen, wodurch die für *Beauveria* typischen Sporenköpfchen zustande kommen. Ausserdem sind die *Beauveria*-Arten alle sich schnell ausbreitende Pilze, welche ein wolliges Myzel oder eine puderige Konidienschicht bilden, wogegen *Tritirachium*-Arten in kleinen, kissenartigen, im Wachstum stark begrenzten Kolonien auftreten. Unser Pilz steht dem *Tr. musea*, dem er in mancher Hinsicht gleicht, sehr nahe, unterscheidet sich von dieser Art jedoch hauptsächlich durch die typische Farbe seiner Kolonien.

Da der Pilz für die nicht sehr artenreiche Gattung neu war, wurde er wie folgt beschrieben:

Tritirachium roseum nov. spec.

Rasen sich sehr langsam ausbreitend, von begrenztem Wachstum, kissenartig mit tiefen Falten, anfangs lila oder braun, oder eine Mischung beider Farben. Im Alter tritt die lila Farbe immer mehr gegen die braune zurück.

Konidienträger aufrecht, wenig verzweigt, $1,7\text{--}2\mu$ dick mit quirlständigen oder opponiert stehenden Aesten.

Aeste gerade, meist 3 an der Zahl, $1,7\text{--}2\mu$ dick und $30\text{--}50\mu$ lang, mit hin und her-gewundenen, zackigen Endauszweigungen.

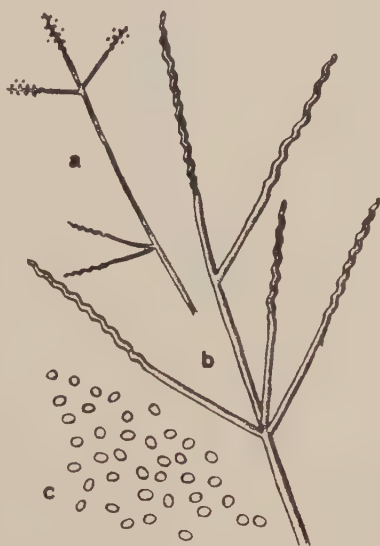


Fig. 8. *Tritirachium roseum*.

a. Konidienträger mit Konidien. Vergr. 245 $\times$.

b. Konidienträger. Vergr. 750 $\times$.

c. Konidien. Vergr. 750 $\times$.

Konidien klein, länglich rund bis subglobos, hyalin, glatt, an den Zacken der Aeste abgeschnürt, fast ährig stehend, meist $(2,3-2,7) \times 2 \mu$ gross.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 20 Tagen: sehr langsam wachsend, Kolonien 1–1,5 cm im Durchmesser, kissenartig, filzig, schön rosa-lilafarben, etwa 494–497–498 mit einigen radiären Falten. Rand scharf, etwas in den Agar hineingewachsen, manchmal daselbst gekerbt. Kein Geruch. Agar nicht gefärbt. Unterseite mit radiären Falten, braun.

Auf Röhrchen nach 20 Tagen:

Auf Bierwürze-Agar: zahlreiche, zum Teil miteinander verwachsene Kolonien, flach hügelig, sammetartig, Farbe 572 mit braun vermischt. Rand scharf. Rückseite braun.

Auf Kirsch-Agar: Kolonien 4–5 mm, flach hügelig, filzig, verschiedenfarbig, 0471 oder 397 mit Rand 222. Rand scharf. Rückseite dunkelbraun.

Auf Möhre: Zahlreiche zum Teil miteinander verwachsene Kolonien, flach hügelig, sammetartig, Farbe 572 mit braun vermischt. Rand scharf. Rückseite braun.

Auf Kartoffel-Agar: Kolonien flach hügelig, sammetartig, etwa 498, überwachsen von braungelbem Myzel. Rückseite braun.

Auf Kartoffelstück: Zahlreiche kleine Kolonien, blassrosa. Das Stück ungefärbt.

Hab. Wahrscheinlich aus Einstreu in einem Kuhstall (HENNEBERG, No. 1316).

Lateinische Beschreibung

Conidiophoris erectis, raro ramosis, crassis $1,7-2 \mu$, in apice coronam 3–4 ramorum gerentibus. Ramis $1,7-2 \mu$ crassis et 30–50 μ longis, in apice serratim curvatos. Conidiis parvis, subglobosis, hyalinis, levibus, $(2,3-2,7) \times 2 \mu$.

Cephalosporium lanoso-niveum nov. spec.

Dieser Pilz wurde von Frl. M. F. HABEKOTTÉ im Laboratorium für Technische Botanik in Delft isoliert aus den Haaren des Baumfarnes *Cibotium Schiedei* Schl. et Cham. Auf Agar-Nährböden bildet er schneeweisse, wollige Myzeldecken, welche auch im Alter ihre Farbe

beibehalten, an der Unterseite jedoch anfangs gelb, später mehr dunkelorange Töne aufweisen. Auf dem Mycelium entstehen meist mehrere kleine, farblose Wassertröpfchen. Der Pilz zeichnet sich vor allen Dingen durch seine kleinen Konidien aus und unterscheidet sich dadurch von allen anderen, ähnlich wachsenden Formen dieser Gattung wie *C. niveo-lanoso* Benedek mit Konidien $5,2 \times 2,6 \mu$ (13), *C. album* Preuss mit Konidien $(3,3-3,4) \times (1-1,5) \mu$ (14) und *C. longisporum* Petch mit Konidien $(6-12) \times (1,5-2,5) \mu$ (15).

Die Beschreibung des Pilzes lautet wie folgt:

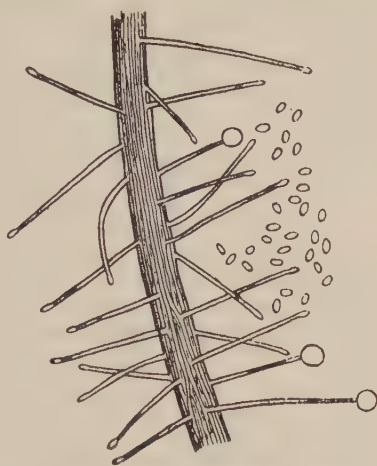


Fig. 9. *Cephalosporium lanoso-niveum*.

Konidienträger und Konidien. Vergr. 750 $\times$.

Cephalosporium lanoso-niveum nov. spec.

Rasen dicht, wollig, weiss, ziemlich schnellwüchsig, aus etwa 2μ dicken Hyphen bestehend, welche sich oft zu $5-7 \mu$ dicken Strängen vereinigen, von denen die Konidienträger allseitig abgehen.

Konidienträger länglich, gerade, $30-35 \mu$ lang, an der Basis $1,3-1,7 \mu$ breit, nach der Spitze zu sich allmählig verjüngend und daselbst ein hyalines, kugeliges Köpfchen bildend.

Konidien zahlreich, hyalin, glatt, kurz-ellipsoidisch, $(2,3-3) \times (1,3-2) \mu$ meist $(2,3-2,7) \times 1,7 \mu$ gross.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 14 Tagen: Kolonie $3,5$ cm im Durchmesser, schneeweiss, wollig mit untiefen radiären Falten, im Zentrum mit einem flachen Hügel. Rand scharf. Ein farbloser Saum fehlt. Kein Geruch. Unterseite mit radiären Falten, 187—182—192.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: Eine locker-wollige, weisse Myzeldecke, an der Unterseite gelb bis gelborange, mit zahlreichen Falten.

Auf Kirsch-Agar: Eine flachwollige, filzige, weisse Myzeldecke mit kleinen, farblosen Wassertröpfchen. Unterseite farblos.

Auf Kartoffelstück und Möhre: Eine filzige, weisse Decke mit grossen, farblosen Wassertropfen.

Auf Sabouraud-Agar: Eine dichtwollige, weisse, dicke Pilzhaut mit zahlreichen Falten. Unterseite rotorange.

Hab. Von den Haaren von *Cibotium Schiedei* Schl. et Cham. (Frl. HABEKOTTÉ).

Lateinische Beschreibung:

Mycelio niveo, lanoso. Capitulis conidiorum globosis, 5—10 diam. Conidiophoris longis 30—35 μ , rectis, in basi 1,3—1,7 μ crassis. Conidii hyalinis, levibus, breviter ellipsoideis, minimis, (2,2—3) $\times$ (1,2—2) μ .

Zusammenfassung.

Es wurden folgende Pilze im Centraalbureau voor Schimmelcultures zu Baarn neu beschrieben: *Arachniotus dankaliense*, *Penicillium ingelheimense*, *Phoma suecica*, *Margarinomyces decumbens*, *Phialophora atra*, *Phialophora cyclaminis*, *Tritirachium cinnamomeum*, *Tritirachium roseum* und *Cephalosporium lanoso-niveum*.

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(From the Microbiological Laboratory, Agricultural University, Wageningen).

MOLYBDENUM AS A GROWTH FACTOR OF *AZOTOBACTER CHROOCOCCUM*

by

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(Received October 18, 1942).

It was BEIJERINCK (1) who was the first to establish that *Azotobacter chroococcum* is a micro-organism having the property to assimilate free nitrogen and use it for its protein synthesis. Since then both microbiologists and agriculturists have paid much attention to this germ. Though the significance of the free-living, nitrogen assimilating bacteria may often have been over-estimated, it is nevertheless probable that under quite special conditions *Azotobacter* may supply important amounts of protein to the soil. Anyhow, agriculture and agricultural science are more than merely theoretically interested in BEIJERINCK's discovery. Surely *Azotobacter* is also of practical importance and so it is not to be wondered at, that *Azotobacter* has been the subject of a great many investigations.

The study of its occurrence in various soils made the great sensitiveness of *Azotobacter* for an acid reaction apparent. It is on this property that CHRISTENSEN (4) founded his method for the indication of an eventual need of liming in Danish soils. This method in a somewhat modified form could later on be used by SÖHNGEN c.s. (10) for the determination of the „Hooghaldisease" (grey speck disease) on the reclaimed moor soils („dalgronden") in Holland. An important factor for the occurrence of this disease turned out to be the level of saturation of the humus complex with bases. This was further substantiated by HUDIG's investigations (5) on the lime status.

Another property characterizing *Azotobacter* is its great need of phosphate. Using this property SACKETT (9) has been able to distinguish 4 groups of soils by means of *Azotobacter* cultures. For this purpose he followed the method, such as it has been worked out by WINOGRADSKY (12): 50 g of soil are mixed with 150 mg K_2SO_4 and 1 g of mannitol

(as a carbon and energy source for the development of *Azotobacter*) and CaCO_3 (to make sure of a suitable alkalinity) and water in a quantity such as to allow the formation of a plastic paste. A small glass dish is supplied with this mixture, and after having been inoculated with a suspension of *Azotobacter* its surface is smoothened. The development of *Azotobacter* on this soil mixture is deemed sufficient for the need of phosphate in the soil. Thus far it has been found as a matter of fact that with the exception of phosphate all essential elements, although not having been supplied intentionally, are present in the soil to a sufficient extent. It has been noted, however, that this is not always the case. Magnesium for instance may be present in so low amounts that the development of *Azotobacter* is prevented. But also the elements, very small quantities of which are needed for nutrition, the so-called „Spurenelemente“ or oligoplerons (*i.e.*, „which make complete in small quantities“) may occur below the minimum and influence its development in that way. One of these elements is molybdenum, which is needed in a nitrogen-free medium.

BORTELS (2) was the first to prove molybdenum as necessary for *Azotobacter*. TER MEULEN (7) made clear that this element may occur in varying quantities in the soil and VAN NIEL (8) demonstrated that some soils contain molybdenum in too low a concentration to allow of a good development of *Azotobacter*. He used the WINOGRADSKY soil plate method for that purpose. BORTELS (3) could confirm this result. He stated that the size of the colonies on soil plates resulting from 2 different soils came up to 4.6 *viz.* 4.34 mm. After supplying 0.0005% *viz.* 0.01% Na-molybdate the average size amounted to 10.0 *viz.* 7.01 mm. VAN NIEL investigated some Californian soils, containing 0.006 to 0.007 mg Mo pro kg soil. After mixing these soils with a Mo-free nutrient solution no development of *Azotobacter* occurred, whilst the supplying of 0.00005% Na_2MoO_4 to the solution resulted in a rich growth; moreover phosphate had to be supplied. In connection with the experiments of BORTELS mention has to be made of the fact that the size of the colonies is largely dependent on their number.

In our investigation we tried to answer the following questions.

- 1°. Is it possible to demonstrate by means of the soil plate method any lack of Mo in the soil of plots occurring in the experimental garden of the Microbiological Laboratory at Wageningen?
- 2°. To which extent can the development of *Azotobacter* be influenced by supplying different quantities of molybdenum to Mo-free liquid cultures or to solid media?

- 3°. The other growth factors being optimal for *Azotobacter*, can a similar variation be induced by means of supplying different quantities of soil, and if so,
- 4°. Is it possible to draw any conclusion on the Mo-content of the soil by comparing these results with the standard series of known Mo-contents?

In our investigation the soils of 4 plots were involved:

Plot 1: A meagre sand soil, loss on combustion 1.2%, pH 5.24;

Plot 8: An acid sand soil, loss on combustion 3.1%, pH 4.82;

Plot 33: A fluvatile clay, rich in lime, pH 7.90;

Lathyrus plot: since 1932 cultivated with *Lathyrus odorata*, loss on combustion 5.15%, pH 5.11. In the latter soil the growth of *Lathyrus* has attained merely a very low level. According to BORTELS (3) *B. radicola* also needs Mo for fixing nitrogen and so the question rose whether this so called „soil sickness” might be connected with a lack of Mo.

1. THE SOIL PLATE METHOD.

The following method has been used. 4 cm glass dishes were supplied with 10 grams of soil, moistened with culture solution and inoculated with *Azotobacter*, so that a layer of about 0.5 cm in height with a smooth surface resulted. Usually 1% CaCO_3 was supplied. These small dishes were placed in glass dishes of a larger size, which had been provided with a layer of moist filter paper in order to avoid desiccating of the soil surface. After 3 days the development of *Azotobacter* colonies on the smooth surface was estimated. As a nutrient medium a solution was adopted, containing pro litre: 20 g mannitol, 1 g K_2HPO_4 , 0.5 g $\text{MgSO}_4 \cdot 7 \text{ aq.}$, 0.2 mg Fe, 0.18 mg Zn, 0.04 mg Cu, 0.02 mg Mg (solution I). Chemicals of purest grade dissolved in water, which had been distilled out of a Pyrex apparatus were used.

Cultures resulting from the 4 soils when treated in the above way, might give the impression that plot 1 and the lathyrus plot would not be able to procure enough Mo to allow the development of *Azotobacter*. In fact these soils either yield no colonies, or only a few appear on each cm^2 . On the soil plates of plot 8 40 to 50 pro cm^2 can be counted, whilst the plate of the clay plot 33 develops many well shaped colonies. When however a control was put up, into which Mo had been supplied, it appeared that 0.5 γ Mo (as Na_2MoO_4) would not result in any improvement. Not even 5 γ Mo pro 10 g of soil could stimulate *Azotobacter* to growth. As according to TER MEULEN (7) fertile soils contain 0.1

to 0.2 mg Mo pro kg, this amount ought to suffice. It was shown moreover that these quantities, mixed with soil from the plots 8 and 33, did not produce any effect on the development of *Azotobacter*. So the reason why *Azotobacter* does not thrive either on soil of plot 1 or of the lathyrus plot cannot be ascribed to lack of molybdenum.

Although it did not seem probable that a lack of lime might induce the poor development, nevertheless we carried out an experiment with an additional supply of lime. Arrangement and results of this experiment are given in table 1.

Table I

	Mo added	CaCO ₃	Development of <i>Azotobacter</i>
Plot 1	0	1%	no or hardly any development
	5 γ	1%	
	0	5%	1 to 2 colonies pro cm ²
	5 γ	5%	
Lathyrus plot	0	1%	3 to 4 colonies pro cm ²
	5 γ	1%	
	0	5%	about 10 colonies pro cm ²
	5 γ	5%	
Plot 8	0	1%	40 to 50 colonies pro cm ²
	5 γ	1%	
	0	5%	60 to 70 colonies pro cm ²
	5 γ	5%	

Though evidently an extra supply of CaCO₃ somewhat stimulated the development, it is not permitted to conclude that a still further supply of lime would induce a development of *Azotobacter* in plot 1 and the lathyrus plot up to the level of plot 8. By repeating the experiment this was confirmed. In the latter experiment half of the plates were supplied with a larger amount of K₂HPO₄ viz. 30 mg pro 10 g of soil, whilst some of the plots were provided with 2% mannitol. None of these extra supplies had any distinct action on the development of *Azotobacter*. On the plates of plot 1 and the lathyrus plot *Azotobacter* develops poorly or not at all, in spite of the additions. On these of plot 8 and the clay plot the development is good or even very good, even if no extra molybdenum, mannitol or K₂HPO₄ had been supplied.

For the moment we are not able to indicate the actual cause of this

phenomenon. We noted, however, that the soil from plot 1 and the lathyrus plot were not easily kneaded into a plastic substance, so that a smooth surface could not be obtained. The soil of plot 8 could easily be handled, and this was still the more so for the soil of the clay plot. The measure in which colonies may develop is strongly connected with these facts. In our opinion it may be due to the lacking of certain soil colloids, which will swell with water. Neither could KELLER (6) ever obtain regular round colonies on sand soils. For that reason he mixed these soils with 10% kaoline.

For the same reason SACKETT and STEWART (9) recommend mixing the sand with 10 or 20% kaoline.

Since pure kaoline cannot be obtained, it could not be put to use in our experiments. So we will have to postpone such experiments until some suitable molybdenum-free plastic material may be available.

2. INFLUENCE OF MOLYBDENUM ON THE DEVELOPMENT OF *Azotobacter* IN LIQUID CULTURES.

As a standard medium solution I (cf. page 125) in a slightly modified form was adopted; the concentration of Fe was changed from 0.2 to 4 mg pro litre. Portions of 20 ml of this solution II in Erlenmeyer flasks of 100 ml were supplied with 0.1 g CaCO_3 . Without any additional molybdenum *Azotobacter* does not develop in this solution. As low an amount as 0.02 γ Mo suffices for some slight growth. Along with increasing amounts of Mo the development increases in a way that a supply of 200 γ Mo provides for the development of a thick coherent film. 2000 γ however causes a decrease in development. The question rose whether the addition of increasing amounts of soil might give rise to similar results. If so the amount of available Mo in soil might be estimated by this method. Experiments with soil from the plots 1 and 8 pointed in this direction, as the results offered in table 2 indicate.

The results of this experiment seem to indicate that plot 8 would contain more Mo than plot 1. Are we however entitled to conclude that a more abundant development as induced by a larger supply of soil may be exclusively ascribed to the presence of more molybdenum? Without further investigation it is not allowed to do so. In fact if with standard solution II provided with an optimal concentration of molybdenum the optimal development might not be arrived at, it might seem very probable that along with the addition of soil other growth factors would be added in increasing amounts. An experiment in which

increasing amounts of soil had been combined with increasing amounts of molybdenum seemed to prove that standard solution II is not optimal.

Table II

Development of *Azotobacter* in 20 ml of standard solution II after 6 days at 28° C.

No.	g CaCO ₃	g soil		Development of <i>Azotobacter</i>
1 and 2	0.1	Plot 1	0.01	no or hardly any development
3 and 4	0.1	„ 1	0.1	slight growth (like 0.02 γ Mo)
5 and 6	0.1	„ 1	1.0	thick, coherent film
7 and 8	0.5	„ 1	5.0	very thick film
9 and 10	0.1	„ 8	0.01	hardly any development
11 and 12	0.1	„ 8	0.1	coherent film (like 2 γ Mo)
13 and 14	0.1	„ 8	1.0	thick, coherent film
15 and 16	0.5	„ 8	5.0	very thick coherent film

If it was optimal, a supply of a small amount of soil and a definite Mo supply would result in a same degree of development as would be induced by a larger soil gift without any additional molybdenum. Generally however this appears not to be the case. The following experimental series was put up:

Soil from plot 8	Mo gifts in γ				
0 g	0	0.2	2	20	200
0.1 g	0	0.2	2	20	200
0.5 g	0	0.2	2	20	200
1.0 g	0	0.2	2	20	200
5.0 g	0	0.2	2	20	200

One striking fact is offered as a result from this experiment: in all of the sets with an equal supply of Mo the development increases along with increasing additions of soil. Merely if the soil would have contained a preposterously large quantity of Mo, such as in the order of 10 γ or more per g, this result might have been ascribed to the action of molybdenum. Hence presumptively still other stimulating substances are included in the soil. Further investigations indicated that the standard solution II might be improved by adding somewhat larger amounts of Cu and Mn. So in further experiments solution III has been used,

containing pro litre: 20 g mannitol, 1 g K_2HPO_4 , 0.5 g $MgSO_4$ 7 aq., 4 mg Fe, 1.8 mg Zn, 0.4 mg Cu and 0.2 mg Mn.

If the soil would contain other growth stimulating minerals, a better growth might be induced by the addition of ash of soil to solution III. To lots of 20 ml of this solution 2 g of soil, c.q. ash of 2 g of soil from the plots 1 and 8 were added. After 4 days at 28° C. a thick film had developed on all cultures provided with soil, whilst in the cultures where ash had been added a merely thin film was formed. So apparently ignition of soil had acted very unfavourably. As it might be that part of the mineral substances had been rendered insoluble by the process of ignition, the experiment was repeated and extended with another series. The latter was supplied with lots of soil which after the ignition had been treated with hot concentrated HCl in order to obtain soluble chlorides. The remaining HCl had been evaporated off with due care. The scheme of the experiment was as follows:

Nos. 1, 2 and 3: 2 g of soil from plots 1, 8 and 34, not ignited;

Nos. 4, 5 and 6: 2 g of soil from plots 1, 8 and 34, ignited;

Nos. 7, 8 and 9: 2 g of soil from plots 1, 8 and 34, ignited, afterwards treated with HCl.

Nos. 1, 2 and 3 all provided for a good growth: in the others, however, the development was markedly lower. Differences between the nos. 4, 5 and 6 on the one hand and nos. 7, 8 and 9 on the other could not be observed.

This experiment leads to the conclusion that organic matter in the soil strongly stimulates *Azotobacter* to growth. In the soil of plot 1 this action is much weaker than in the soil of plot 8: this is clearly shown when lower amounts of soil 1 have been added (see page 128). This difference is doubtlessly caused by the large difference in humus content of these soils.

Subsequently we investigated whether the growth stimulating substances might be removed out of the soil, either by treating the latter with H_2O_2 or by extraction with 25 ml of 0.1 n KOH per 5 g of soil. Into each culture 2 g of treated c.q. untreated soil were added. For the soil of plot 1 these treatments had hardly any effect. The clay soil from plot 34 could not be extracted with KOH in this way. So we were obliged to abandon the treatment with an alcali of the latter soil. In this experiment it was noteworthy that the stimulating action of plot 34 had markedly increased by the treatment with H_2O_2 . We are not able to offer an explanation for this phenomenon. Initially the same phenomenon seemed to be induced by the soil of plot 8 as well. After incubating

8 days at 28° C. the supply of untreated soil, however, had resulted in a thick film, whilst the soil treated with H_2O_2 on the contrary had merely induced the formation of a thin one. Extraction with KOH also had acted obviously unfavourably on this soil. Addition of a HCl-precipitate of the humus extract to this culture resulted in considerable improvement. These experiments substantiate, at least partly, the theory of the presence of organic growth substance in the soil. As has been stated before, the mode of action of a treatment with H_2O_2 on clay soil is not yet clear.

Moreover these experiments have indicated that the optimal culture medium for *Azotobacter* is by no means definitely known as yet. We cannot, however, start an investigation into the need of molybdenum when we are as yet unable to grow *Azotobacter* under definitely known optimal conditions.

3. INFLUENCE OF MO AND CU ON THE DEVELOPMENT OF *Azotobacter* ON SOLID CULTURE MEDIA.

It is known that *Azotobacter* will grow better on solid media than on solutions. Difficulties resulting from the formation of a film in the solutions do not occur here. For the problems here at hand it is a drawback, however, that solid media cannot easily be freed from minute quantities of organic and inorganic matter. Out of silicagel as well the removal of the last traces of minerals causes difficulties. Even after dialysis during a longer period against quite pure distilled water, those traces cannot be wholly removed. It is known that silicagel stimulates the growth of *Azotobacter* (SÖHNGEN (11)). It is not known, however, whether this growth stimulation is due to the colloidal state of silicagel or to the occurrence of impurities.

Experiments with carefully washed out agar did not proceed satisfactorily. *Azotobacter* developed poorly; addition of various amounts of Mo, Fe and Cu did not result in any improvement. Ash of soil extract ameliorated the growth in a small measure, whilst untreated soil extract caused an abundant growth. The favourable development in the latter case is apparently due to the presence of organic matter in the extract.

A much better development was obtained on silicagel-plates according to WINOGRADSKY. A mixture of 12.5 ml Na silicate (9° Bé) and 12.5 ml HCl (13° Bé) was poured in 9 cm Petri dishes. After 24 hours the gel had solidified. The superfluous HCl was removed by washing out in streaming tap water. Further purification was carried out by

a submersion during 4 days in very pure distilled water. Initially during two days the water was renewed twice daily, afterwards once.

After drying the plate surface of the silicagel on a hot water stove the surface was evenly moistened with solution III. Moreover all plates were supplied with 2.5 mg Na_2CO_3 . As soon as the solution had been taken up by the gel the plates were inoculated with a young culture of *Azotobacter*. Two of these plates did not obtain any Mo, two others 25 γ each. The following result was obtained:

after 2 days at 28° C.,	0 γ Mo:	good development.
„ 2 „ „ „	25 γ Mo:	very good development.
„ 3 „ „ „	0 γ Mo:	brown.
„ 3 „ „ „	25 γ Mo:	not brown, abundant growth.
„ 6 „ „ „	0 γ Mo:	brown.
„ 6 „ „ „	25 γ Mo:	not yet brown.
„ 12 „ „ „	0 γ Mo:	brown.
„ 12 „ „ „	25 γ Mo:	not brown, a little grayish.

So a very marked difference in development occurs between cultures with 0 and 25 γ Mo. The early changing of the colour into brown in the absence of Mo goes together with an early stagnation in development. The brown coloration is a characteristic property of old *Azotobacter* cultures. It is usually ascribed to the formation of humuslike substances (melanines) probably originating from the oxydation of nitrogen compounds. If this presumption is right, this may also explain why the brown colour does not appear in the presence of Mo. This element with its reductive properties will prevent the formation of the brown substances. On the other hand copper is likely to cause the opposite effect as it stimulates oxydation. This being admitted it will be possible to compose a colour scale for various levels of molybdenum in which the occurrence of various shades will be determined by the amounts of Cu ions present.

In fact results of further investigations confirmed this view. We studied 8 different levels of Mo, namely 0, 0.02, 0.06, 0.2, 0.6, 2, 6 and 20 γ Mo supplied to a plate and combined with 0.00004% Cu or 0.000004% Cu. The results agreed with our expectation. Both sets yielded a colour scale varying from dark brown to colourless. When the higher amount of Cu had been supplied, however, a larger supply of Mo was required to obtain a colourless culture. Figures 1 and 2 may give an impression of these phenomena, although the actual differences were more distinct than in black and white of the photo.

The latter experiments substantiate the theory that assimilation of the free nitrogen is to be understood as a reduction process. Molyb-

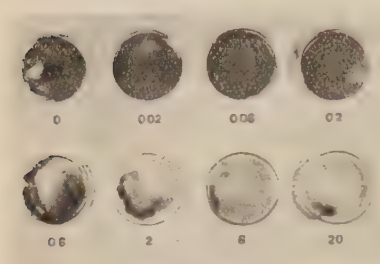


Fig. 1.

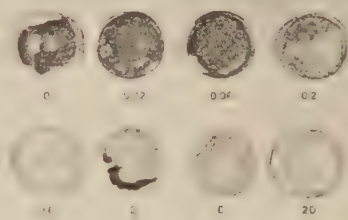


Fig. 2.

Azotobacter cultures on silicagel-plates with Mo gifts varying from 0 to 20 γ ; photo 1 with 0.00004% Cu, photo 2 with 0.000004% Cu.

denum acts here as a katalysator. We did not try to decide whether NH_3 or NH_2OH are the intermediate products. It is no easy matter to prove the formation of such intermediate substances, as it will remain very difficult to distinguish between formation of such substances by the primary reduction of free nitrogen or by the dissimilation of cell-protein.

Mention should be made of our observation that small amounts of Mo may stimulate the assimilation of nitrate-N by *Azotobacter* as well. This is made evident by an experiment, in which two sets of cultures in standard solution III were put up. Each flask with 20 ml was provided

with 0.1 g CaCO_3 and increasing amounts of Mo (0, 0.2, 2 and 20 γ). To one of these sets 0.1% KNO_3 had been added.

The set without nitrate yielded similar results as were obtained in former experiments of the same character: larger supplies of Mo always induced a better deve-

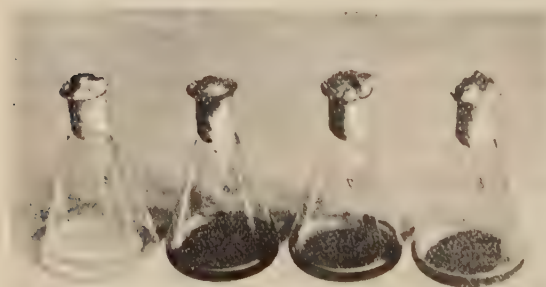


Fig. 3. Liquid cultures of *Azotobacter chroococcum* with 0.1% KNO_3 and 0, 0.2, 2 and 20 γ Mo.

lopment and in the absence of Mo hardly any growth could be noted. A same absence of growth occurred when Mo was lacking in a culture containing nitrate; 0.2 γ Mo in this set however produced a good development. In the nitrate set, however, the cultures provided with 2 and 20 γ Mo were hardly any better developed than the one with 0.2 γ (Figure 3). Probably for the assimilation of nitrate-N *Azotobacter* does not need as much Mo as is required for the assimilation of the atmospheric nitrogen. This assumption does not seem farfetched as nitrate-N can be reduced more easily into the nitrogen of protein than the nitrogen molecule which is slower in reacting.

Summary and conclusions.

Though the discovery of molybdenum as an element necessary for the growth of *Azotobacter* enables us to cultivate this organism in a purely synthetic medium, free of combined nitrogen, the optimal medium for its development is not known as yet. Evidence is given that organic matter of the soil is highly favourable.

Molybdenum acts as a reductor in the assimilation of atmospheric nitrogen. For the reduction of nitrate nitrogen there is needed less Mo than for the reduction of free nitrogen.

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SALMONELLA ANTIGENS IN BACTERIUM COLI AND PARAGGLUTINATION

by

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In co-operation with other workers KUHN (15, 16, 17) described as paragglutination the phenomenon that a.o. *Bacterium coli* from the intestines of patients suffering from dysentery or typhoid fever were sometimes agglutinated by dysentery or typhoid sera, contrary to *B. coli* from the intestines of healthy persons. This paragglutination differed from co-agglutination in as far as the property in question was lost in sub-cultures. They explained this by assuming that *B. coli* had become susceptible to the specific agglutinins against the dysentery bacillus or *B. typhosum* owing to the growth in the intestines of patients, a susceptibility which disappeared again under normal conditions.

The phenomenon was confirmed by other workers (RIMPAU (18), GAETHGENS (8), DITTHORN and NEUMARK (6), BUSSON (4)), but some called it in question (a.o. ARKWRIGHT (1)) and others totally rejected it (BREINL (3)).

In the past few years various workers described *B. coli* showing antigens that are also found with representatives of the *Salmonella* group (HABS and ARJONA (11), GARD (9), GARD and ERIKSSON (10), BRAUN, SILBERSTEIN and ÜLKER (2)), which bacteria were called „*Salmonella coli*" by KAUFFMANN (12, 13). According to him the conception paragglutination is superfluous, as it is not to be distinguished from the usual agglutination. The agglutination of some *B. coli* by typhoid or dysentery sera should be attributed to the presence of antigens in common that these bacteria cannot obtain in the intestines of patients, but must possess already beforehand and the disappearance of this agglutination in sub-cultures must be due either to the loss of the antigens, to the transformation into rough-antigens or to other variability.

With a view to these different opinions we thought it of importance to reinvestigate this problem.

For this purpose we isolated *B. coli* from the faeces of 100 patients suffering from typhoid or paratyphoid fever, as well as *B. coli* from the faeces of 90 healthy persons, who had not suffered from either of these two diseases. The occurrence of paragglutination was ascertained by means of a typhoid and a paratyphoid B serum, whilst the presence of *Salmonella* antigens was tested by using O-, Vi- and H-sera.

Table I

No. of cultures	Paragglutination with		<i>Salmonella</i> antigens found	Infection with
	Ty serum	Pty B serum		
2350	—	—	Vi	<i>S. typhi</i>
2502	—	—	IV, VII, XXV	<i>S. typhi</i>
2861	—	—	Vi	<i>S. paratyphi</i> B
2892	+ 1 : 80	—	(I), VI, XIII, XIV, XXVIII, Vi	<i>S. typhi</i> murium
3162	—	—	Vi	<i>S. typhi</i>
4535	—	—	OA ¹⁾	<i>S. paratyphi</i> B
4589	—	—	OA ¹⁾	<i>S. typhi</i>
6431	—	—	XXIX, Vi	<i>S. paratyphi</i> B
6530	+ 1 : 160	—	XXVIII, Vi	<i>S. typhi</i>
6768	—	—	Vi	<i>S. typhi</i>
6822	—	—	Vi	<i>S. typhi</i>
89 cultures	—	—	—	<i>S. typhi</i> or <i>S. paratyphi</i>

¹⁾ In these cases the antigenic formule could not be ascertained more exactly, as after 1 or 2 sub-cultures the antigens had disappeared. OA, OB and OC: mixed diagnostic *Salmonella* O-sera of the International *Salmonella* Centre.

On examining the results of the experiment we see that with the 100 patients (table I) the paragglutination could be observed twice and that with the 90 healthy persons (table II) the same phenomenon occurred equally twice. It was striking that in all these 4 cases *B. coli* possessed *Salmonella* antigens and that they possessed the antigen XXVIII in common. From these facts alone, however, the paragglutination with the typhoid serum could initially not be explained but afterwards we found that our agglutinating typhoid serum possessed agglutinins against antigen XXVIII. As a matter of fact this appeared

Table II

No. of cultures	Paragglutination with		<i>Salmonella</i> antigens found
	Ty serum	Pty B serum	
504	—	—	OA ¹⁾
510	—	—	OB ¹⁾
514	—	—	OA ¹⁾
515	—	—	OC ¹⁾
530	—	—	OA ¹⁾ , Vi
533	—	—	OA ¹⁾
534	—	—	OC ¹⁾
535	—	—	OA ¹⁾ , Vi
553	—	—	Vi
560	—	—	OC ¹⁾
569	+ 1 : 160	—	VI, VII, XIV, XXII, XXVIII, Vi
570	+ 1 : 160	—	(IV), VI, VII, XIV, XXII, XXVIII, Vi
578	—	—	OA ¹⁾ , Vi
77 cultures	—	—	—

¹⁾ In these cases the antigenic formule could not be ascertained more exactly, as after 1 or 2 sub-cultures the antigens had disappeared. OA, OB and OC: mixed diagnostic *Salmonella* O-sera of the International *Salmonella* Centre

to be not so very strange, as from 10 tested fresh strains of *B. typhosum* 3 possessed the antigen XXVIII besides the antigens IX, XII, Vi and d, indicated by KAUFFMANN (13). After saturating the typhoid serum with *S. tel aviv* (XXVIII) we could ascertain that the paragglutination with the 4 strains of *B. coli* had equally disappeared.

So in our cases paragglutination is a usual co-agglutination caused by the possession of an antigen in common. This antigen is not obtained in the intestines of the patient, as *B. coli* isolated from healthy persons may equally possess same. Besides it may be found with *B. coli* emanating from patients infected by a bacillus that does not possess that antigen itself (see culture 2892, table I).

As can be seen from our tables, the occurrence of *Salmonella* antigens with *B. coli* may be widely spread and they may be found in many combinations. As they prove to be present as well with *B. coli* from healthy persons, the conditions under which the bacteria grow do not play any part in this respect and the said antigens must simply be

considered as a property of *B. coli* as such¹⁾. During our experiment we only found *Salmonella* O- and Vi-antigens, but no *Salmonella* H-antigens (just as KRISTENSEN, BOJLÉN and KJAER (14)); so we are under the impression that the H-antigens are more specific for the *Salmonella* than the O-antigens.

It is known that man can lodge more than one strain of *B. coli* at the same time and so it often occurred that not all *B. coli* from one sample of faeces appeared to possess *Salmonella* antigens, so that we had always to examine various colonies. The cultures 2502 and 6530 (table I) concern *B. coli* isolated from the same typhoid patient at different times, which proves that in the intestines also *B. coli* with different *Salmonella* antigens may be found together.

Upon a closer examination of the tables I and II we see that the Vi-antigen, either in combination with the O-antigen or not, is found rather often with *B. coli* (7—8%). As known, Vi-agglutinins are found in the blood of typhoid patients and of carriers of *B. typhosum*, but FAARUP (7) and DAVIS (5) could also prove the presence of these Vi-agglutinins in the blood of 5—8% of healthy, not-inoculated persons.

In a further essay we shall try to answer the question, whether there is actually a connection between the Vi-agglutinins in the blood and the presence of Vi-antigen with *B. coli* from the intestines of healthy individuals.

S u m m a r y.

The phenomenon of paragglutination with typhoid serum of *B. coli* from the intestines of healthy persons and of patients suffering from typhoid or paratyphoid fever was observed and studied. Evidence could be obtained that the paragglutination was to be attributed to the presence of a common *Salmonella* antigen (XXVIII), so that it is wrong to make a distinction between paragglutination and co-agglutination.

Salmonella antigens were found in various combinations with *B. coli* and different combinations could also be ascertained with the same person.

The attention is drawn to the presence of the Vi-antigen with *B. coli*, also from healthy persons, and the possibility of its connection with the Vi-agglutinin in the blood of healthy persons is pointed at.

¹⁾ As we isolated from a kidney of a cow a strain of *B. coli* which shows paragglutination and also possessed the antigen XXVIII, it is proved, that *Salmonella* antigens can also be found in *B. coli* from other origin.

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ON THE ACTION OF SULFANILAMIDE VIII. THE MECHANISM OF ITS ACTION ¹⁾

by

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1. INTRODUCTION.

The action of sulfanilamide and related drugs differs widely from that of germicides in general. This was already evident in the early study of these drugs, as an action on streptococci in vitro seemed to be absent. Soon it became obvious however, that the substance was active in vitro but only when inocula were small (2). Later it was demonstrated that sulfanilamide worked solely on actively growing bacteria, its action only becoming manifest after some apparently normal cell divisions (25). This „lag-time of action” puts sulfanilamide quite apart from ordinary desinfectants and marked it as a special kind of growth inhibitor.

Thus sulfanilamide not being directly bactericidal nor bacteriostatic in the usual sense, many authors have already supposed an action of the drug on bacterial metabolism f.i. on some bacterial enzyme.

LOCKE, MAIN and MELLON (17) supposed that sulfanilamide acts on bacterial catalase. The growth inhibition then would be due to the resulting accumulation of H_2O_2 . In our opinion however this hypothesis can not offer the clue for the mechanism of its action (12).

In previous papers we gave the results of some of our systematically proceeding steps to obtain evidence about the part of bacterial metabolism sulfanilamide interferes with. We first studied respiration (23, 24) and we could not observe any influence of sulfanilamide on O_2 consumption by bacteria as measured with WARBURG's technique, nor on bacterial dehydrogenation. We are aware that these results are not in all respects in agreement with those of some other investiga-

¹⁾ Seventh communication: K. C. WINKLER and H. W. JULIUS, *Antonie van Leeuwenhoek* 8, 86, 1942.

tors (7, 10, 16). To explain this is not within the scope of this paper, but since it is evident that, under conditions in which sulfanilamide can pursue its action on growing bacteria, action on bacterial respiration can be absent, neither here the explanation of its actual mechanism can be found.

Moreover the work of BARKER (1), GIESBERGER (8) and CLIFTON (4) made it probable that the lack of any influence on carbohydrate katabolism excludes the possible synthesis of carbohydrates by bacteria as well. Thus it seems improbable that the explanation of sulfanilamide action might be attributed to bacterial carbohydrate metabolism in general.

These experiments lead us forcibly to the supposition that the nitrogen metabolism in its widest sense or — though very improbable — the fat metabolism can offer the only points of attack of the sulfanilamide action. Experiments in this line of thought are in progress.

In 1940 WOODS (26) revealed the important fact that a substance in yeast extract could counteract sulfanilamide and restore bacterial growth in cultures containing sulfanilamide. This substance was supposed to be identical with p. aminobenzoic acid.

In further work by FILDES (6), STAMP (22) and GREEN (9) it was suggested that sulfanilamide inactivates an essential coenzymic grouping of the susceptible organism and that this grouping is most probably p. aminobenzoic acid.

Shortly afterwards RUBBO and GILLESPIE (20) showed that p. aminobenzoic acid was a growth factor for *Cl. acetobutylicum* and lately KUHN and SCHWARZ (14) showed the same for *Streptobacterium plantarum*.

The general inference at this time appears to be that the growth factor p. aminobenzoic acid is the prosthetic grouping of an essential bacterial ferment. The equilibrium apoferment + p. aminobenzoic acid $\rightleftharpoons$ holoferment is disturbed by sulfanilamide, which, by its structural analogy, is adsorbed or bound to the apoferment. The sulfanilamide containing ferment is supposed to be inactive and the growth of the micro-organism is retarded or brought to a standstill. Other combinations similar to sulfanilamide — p. aminobenzoic acid have been found to exist (15).

We will now try to expound our views on the mechanism of sulfanilamide action dealing first with the p. aminobenzoic acid hypothesis as several arguments in connection with this very alluring theory suggest (in our opinion) that the mechanism might not be as simple as that.

2. DISCUSSION.

A. a. p. Aminobenzoic acid in very small concentrations already inhibits the action of sulfanilamide. It is even claimed (20) that one molecule of p. aminobenzoic acid inhibits the action of 23000 molecules of sulfanilamide on *Cl. acetobutylicum*! In our own experiments we found this ratio to be 1 : 10000 for streptococci and for *B. coli* 1 : 1200. It must however be kept in mind that the calculated ratio is based on concentrations in the media. On their actual place of action, only one molecule p. aminobenzoic acid can compete with one molecule of sulfanilamide and vice versa. This suggests that p. aminobenzoic acid may be strongly adsorbed by the bacteria while sulfanilamide is not. In the experimental part (Exp. VII A and B) it will be shown that as a matter of fact p. aminobenzoic acid is strongly adsorbed by *B. coli*, whilst sulfanilamide is not adsorbed at all or at best in quantities within the experimental error. This explains in a simple way that it is not necessary to attribute the property of p. aminobenzoic acid to inhibit strongly the action of sulfanilamide to any specific capacity and it reduces the antagonism to mere physico-chemical laws.

This strong adsorption might be explained by arguing that a prosthetic grouping will be strongly „adsorbed” to its apoferment. Now it is clear from fig. 6 that the adsorption process is continuous and follows FREUNDLICH's adsorption isotherm. It can be calculated that in the highest concentration (table II) 10^8 molecules are adsorbed per bacterium. This amount is so much above the quantity that is necessary in growth experiments that it becomes highly improbable that this adsorption can be due to the apoferment only. Besides in this concentration p. aminobenzoic acid is an effective antiseptic for *B. coli*. This property can not possibly be explained by the adsorption of the substance to its apoferment. So it seems to us that the substrate by which the p. aminobenzoic acid is adsorbed cannot be the apoferment.

On the other hand the adsorption must be due to some vital part of the bacteria as a suspension of bacteria heated to 60° C. loses its adsorptive action as soon as the viable count drops below 10% of the original value, i.e., as soon as the adsorption comes within the experimental error (Exp. VII C). Furthermore the adsorption is due to some part which is characteristic for bacteria as p. aminobenzoic acid is not adsorbed to liver cells or red blood cells. Dr A. TH. KNOPPERS (Laboratory of Pharmacology, University of Amsterdam, personal communication, to be published (13)) found that the antipyretic action of sulfathiazol could not be counteracted by p. aminobenzoic acid. All this is in full

agreement with our original assumption that the point of attack of the sulfonamide drugs must be some special complex in the bacteria in which they differ from animal cells.

Thus apart from being a growth factor, p. aminobenzoic acid is adsorbed to some vital part of the bacterium and in higher concentrations it is an antiseptic. Evidently p. aminobenzoic acid acts in two, perhaps in more, different ways; the importance of this fact will be discussed under **A. d.**

So the counteraction of sulfanilamide by p. aminobenzoic acid, strong though it may seem, need not be specific but can be explained by adsorption phenomena.

A. b. p. Aminobenzoic acid is not the only substance inhibiting the action of sulfanilamide. We could show for instance that cysteine has this property in some instances (vid. Exp. II, fig. 2). The fact that this inhibition occurs in about aequimolecular concentration with sulfanilamide does not necessarily prove that its mechanism would differ from that of p. aminobenzoic acid. The strong adsorption of the latter may readily explain this quantitative difference.

There is another substance which strongly inhibits sulfanilamide, *i.e.*, peptone. Its antagonistic action is generally imputed to its supposed p. aminobenzoic acid content. According to Exp. III, fig. 3 peptone shows a marked inhibition of sulfanilamide action in dilutions up to 0.002%, whilst in higher dilutions (0.0002%) inhibition is absent. This experiment moreover shows that the inhibition by peptone between 0.2% and 0.002% is remarkably constant, whilst the counteracting faculty of p. aminobenzoic acid varies with the concentration in the way to be read from fig. 4. With 2% peptone an additional and in fact more complete inhibition is obtained. These facts seem to render the supposition reasonable that two inhibitory substances are present in peptone. Moreover a colorimetric „determination” of p. aminobenzoic acid in 10% peptone water after the method of diazotization and a coupling to oxychinoline shows a colour corresponding with 0.05 m. mol. p. aminobenzoic acid (Exp. V). The actual concentration, however, must be considerably lower as most of the colour produced will be probably due to the tryptophane and similar substances in the peptone. So it is evident from the figures 3 and 4 and from a calculation on the base of the data just mentioned, that peptone inhibits sulfanilamide at least about 150 times more strongly than would correspond with its p. aminobenzoic acid content! It might be supposed that only the ad-

ditional inhibition, apparent in 2% peptone, is due to its p. aminobenzoic acid (cf. fig. 3). Furthermore in some media, containing peptone, sulfanilamide is actively bacteriostatic (Exp. VI, fig. 5) whilst p. aminobenzoic acid still is counteracting sulfanilamide in very low concentrations. This would be impossible if p. aminobenzoic acid was present in peptone in a corresponding amount. The inhibiting action of peptone thus cannot possibly be due to p. aminobenzoic acid. Recently BLISS and LONG (3) have shown that also methionine, in itself an antiseptic, has an antibacteriostatic action towards sulfanilamide. The concentration can be about 1/700 of that of the sulfonamide; it has no growth promoting capacities.

The fact that there are more than one and probably several substances paralysing sulfanilamide action is in our opinion directly contradictory to the p. aminobenzoic acid hypothesis.

KUHN (15) has drawn the conclusion from his experiments on *Streptobacterium plantarum* that the growth promoting properties of p. aminobenzoic acid are annihilated by sulfanilamide and growth is re-established by the addition of p. aminobenzoic acid. Of course any antiseptic will destroy the growth promoting properties of p. aminobenzoic acid, so at the end the primary feature of this remarkable antagonism can only be the capacity of p. aminobenzoic acid to neutralise sulfanilamide! By tacitly supposing p. aminobenzoic acid to be the only inhibitor and by identifying the growth promoting and the inhibiting properties of p. aminobenzoic acid the specific character of the counteraction of this substance is presented as probable. The finding of other substances counteracting sulfanilamide, presumably through other mechanisms, however suggests that the identification of growth promoting and inhibiting properties is not permitted and takes away the strongest argument for the hypothesis.

A. c. In many cases bacteria are actively growing in media in which no p. aminobenzoic acid is present at all, whilst sulfanilamide is markedly bacteriostatic (vid. Exp. I, fig. 1). In the medium used in the experiments of fig. 1 p. aminobenzoic acid may be safely considered as absent. The presence of p. aminobenzoic acid in peptone, as we have just pointed out, is problematical to say the least and the same holds for the blood in Exp. VI. So there seem to exist a great many media devoid of p. aminobenzoic acid and quite a number of bacteria, which do not want p. aminobenzoic acid as a growth factor, whilst sulfanilamide acts nevertheless as a bacteriostatic. This might be explained by sup-

posing a synthesis of the „indispensable” substance by these bacteria (18). This accessory hypothesis weakens however the initial argument for the p. aminobenzoic acid hypothesis, because it is no longer possible to accept p. aminobenzoic acid as a growth factor *sensu strictiori* and the substance takes on the character of a normal product of bacterial metabolism. The accessory hypothesis, however, may be convenient to explain resistance against sulfanilamide as observed by many authors and the relatively high concentration of the drug, which is needed in some cases (fig. 1).

A. d. If p. aminobenzoic acid counteracts sulfanilamide, this does not necessarily mean that the sulfanilamide acts by counteracting p. aminobenzoic acid. In other words it is not necessary to assume an interrelation between the growth promoting and the sulfanilamide inhibiting properties of p. aminobenzoic acid. One substance can have two quite different actions, both of which can even be specific. As to p. aminobenzoic acid there is a case in point:

MANN and KEILIN (19) observed that sulfanilamide specifically inhibits carbonic acid-anhydrase. This property of the substance is by no means linked with its antibacterial activity, the more so as the enzyme is not present in lower organisms. The authors named, moreover, have pointed out that the mechanism is quite different, for in the first place the SO_2NH_2 group is the active grouping in this respect. Benzenesulfonamide and even SO_2NH_2 and $\text{NH}_2\text{SO}_2\text{NH}_2$ are active in this reaction, whilst sulfapyridine and other growth inhibitors with substituted SO_2NH_2 grouping are not. The activity of sulfanilamide as a growth inhibitor depends on the p. amino grouping; essential substitutions here result in a loss of activity. Thus the bacteriostatic action of sulfanilamide and its inhibition of the carbonic acid-anhydrase are two different but specific actions of the same molecule.

In this connection it is important that, according to Dr H. VAN GOOR (Physiological Laboratory of the University, personal communication) p. aminobenzoic acid, at least partially, counteracts the inhibition by sulfanilamide of the carbonic acid-anhydrase of blood *in vitro*. It is highly improbable that p. aminobenzoic acid might be a prosthetic grouping for this enzyme as well as for the enzyme involved in bacterial growth. But even if it were, it then seems impossible to understand why sulfapyridine (etc.) would not inhibit the carbonic acid-anhydrase, nor why $\text{NH}_2\text{SO}_2\text{NH}_2$ would not inhibit bacterial growth. From this we may conclude that p. aminobenzoic acid will compete with sulfanil-

amide on two different substrates and while acting through two different modes. The p. aminobenzoic acid molecule, without being a prosthetic grouping might, by means of its closely related structure, supplant the sulfanilamide in both cases and, missing the strongly polar SO_2NHR grouping, miss the disturbing properties of sulfanilamide in the one case and of sulfanilamide and related substances in the other.

A. e. Finally we must refer to two other interesting facts:

According to FELDT (5) the action of sulfanilamide on *Spirochaeta recurrentis* is not neutralised by p. aminobenzoic acid. This means that, as the action of sulfanilamide in this one case is not due to an antagonism towards p. aminobenzoic acid, in all other cases this can not be the essential mechanism either. It might be supposed of course that sulfanilamide acts along another mechanism on this special organism, but it seems much more reasonable to impute the failure of the „normal“ antagonism to some property of p. aminobenzoic acid itself in relation to the spirochaeta (f.i. lack of adsorption?).

SCHREUSS (21) has shown that marfanil (p. methylaminobenzene sulfonamide) known as a powerful drug against clostridia but also against other micro-organisms, is not counteracted by p. aminobenzoic acid. Again we have to choose between two possibilities. Either marfanil acts in another mode, or, the mechanism of its action is essentially the same as that of all other sulfonamide substances. In the former case the substance does not belong to the sulfonamide drugs and can be left out of our actual considerations. If the latter assumption was true it would furnish one of the strongest arguments against the p. aminobenzoic acid hypothesis. SCHREUSS, to our great astonishment, chooses an explanation between these two alternatives. He accepts a mechanism analogous to that of sulfanilamide, differing only in so far as that there would be, supposedly, another growth factor (p. methylaminobenzoic acid) marfanil interferes with; but he does not give any actual fact in support of this twofold supposition.

B. Considering all this it seems to us that the theory of KUHN and others, alluring though it may be, is not so well founded as to exclude other possibilities to explain the many facts observed in relation to the sulfanilamide action.

We still consider it of the greatest importance to continue investigation on which part of the bacterial metabolism sulfanilamide exerts its action. For even supposing that sulfanilamide supplants the prosthetic grouping of any enzyme (may this be p. aminobenzoic acid or not) it

must be essential for the gaining of deeper insight to know in which part of the bacterial metabolism this presumed enzyme intervenes. On the other hand, if the drug is not interfering with an enzymatic system, the knowledge of the part of bacterial metabolism it acts upon may give the clue to its mechanism.

Above we have presented the considerations which have led us to the assumption that sulfanilamide possibly interferes with bacterial protein metabolism and, in fact as we hope to be able to show in a further communication, with protein synthesis. Within this scope there are some alternatives to be considered.

1. *p*. Aminobenzoic acid might be an essential amino acid for protein assimilation, which — by the way — might also explain its growth promoting properties. The synthesis of bacterial protein would be brought to a standstill when instead of *p*. aminobenzoic acid sulfanilamide is incorporated. The cyclic amino acids are rather scanty in nature. As sulfanilamide is poisonous to bacteria and not in a comparable degree to animal cells this assumption has some base.

2. Sulfanilamide might act on some enzymatic grouping in the micro-organism whilst *p*. aminobenzoic acid might neutralise it only by supplanting the sulfanilamide without itself disturbing the function (electric field) of this active grouping. The strong adsorption of *p*. aminobenzoic acid as compared with sulfanilamide (Exp. VII) might explain why such small concentrations of *p*. aminobenzoic acid act so strongly in this respect.

3. Sulfanilamide might cause an essential disturbance of bacterial protein synthesis, either enzymatic, physico-chemical or by pure chemical bonds. But within the wide range of bacterial adaptation the metabolism has at its disposal still other ways for synthesis on condition that other essentials are within its reach. *p*. Aminobenzoic acid and other amino acids may open the way to an alternative mode of synthesis.

We hope to forward in a further communication some experimental data in favour of an assumption that the mass of bacterial substance with which sulfanilamide or its heterocyclic derivatives and also *p*. aminobenzoic acid react, is relatively large as compared to the total bacterial mass. Again this points more to one of the above offered alternatives than to the disturbance of a specific prosthetic grouping of an enzyme which can be no more than a minute part of the total bacterial mass.

3. EXPERIMENTAL PART.

Experiment I. *B. coli* was grown in a synthetic medium, consisting of 1 g K_2HPO_4 , 1 g $(NH_4)_2SO_4$ and 1 g Na pyruvate in 1000 ml tap water. For the experiments the medium was prepared with only 600 ml tap water and to 3 ml of this stock medium 2 ml of solutions in tap water of the substances to be studied were then added, making the final concentration of the medium as described above.

With our usual technique (11) hourly determinations of the number of viable bacteria in cultures with various sulfanilamide concentrations (0.01, 0.02, 0.05 and 0.1%) were made. The growth curves derived from these data are given in fig. 1. In the ordinate the log of count is given per 28 mg of the culture. To obtain the log of count per ml log 35 should be added.

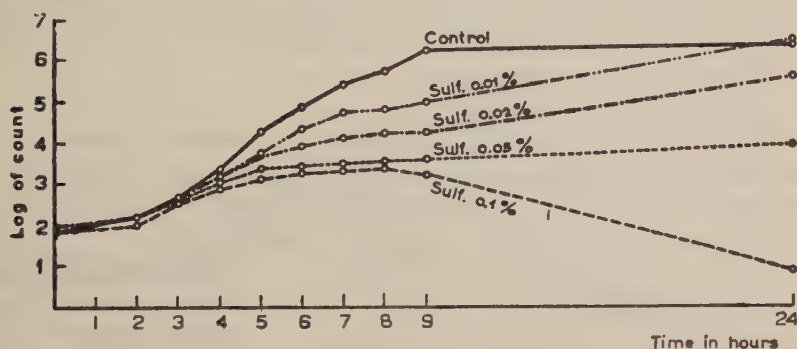


Fig. 1. Growth curves of *B. coli* grown in a synthetic medium with various concentrations of sulfanilamide.

Experiment II. *Streptococcus haemolyticus* (strain ARONSON) was cultured in infusion broth with 50% horse blood. Sulfanilamide was added to a final concentration of 0.015% (= 0.9 m.mol.). The influence of cysteine was studied by adding 0.02% (= 1.4 m.mol.) of this amino acid. The antagonizing action of cysteine in a concentration about aequimolair with sulfanilamide is evident from fig. 2.

Experiment III. *B. coli* was grown in the synthetic medium mentioned in Exp. I. To 3 ml of the concentrated medium 1 ml peptone in various concentrations was added, together with 1 ml sulfanilamide solution or water to obtain the final volume. The inhibition of sulfanilamide by peptone is evident from fig. 3.

The inhibiting substance is active in a concentration of 0.002% peptone. An increase of the peptone concentration up to 0.2% leaves

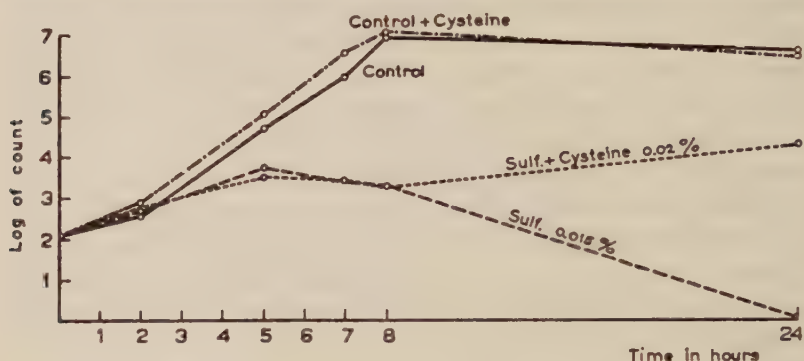


Fig. 2. Growth curves of *Streptococcus haemolyticus* grown in infusion broth with 50% horse blood with sulfanilamide, cysteine and with sulfanilamide + cysteine.

the inhibition unaltered. With 2% peptone an additional inhibition is obtained. In our views this inhibition must be due to a second substance as it is improbable that 100 times the minimal concentration of

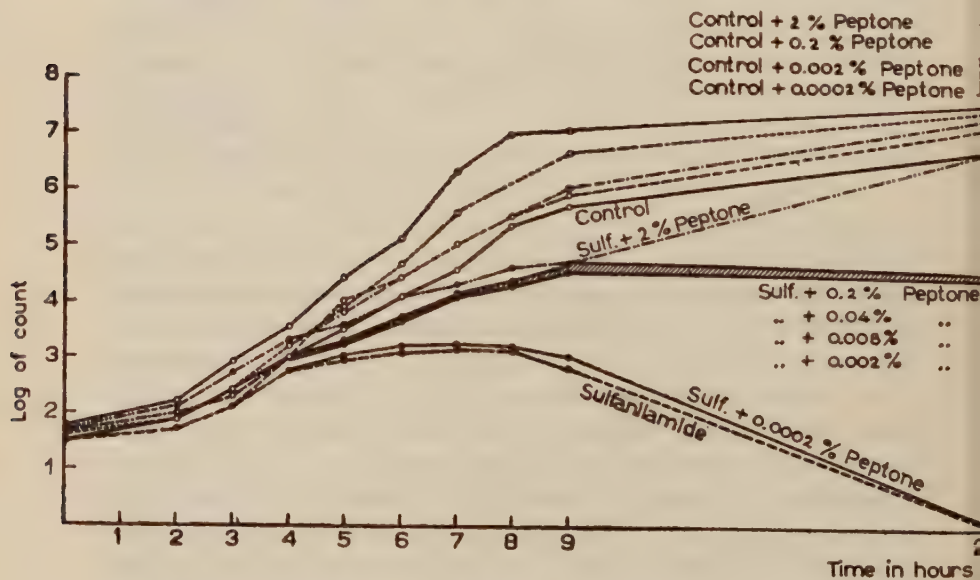


Fig. 3. Growth curves of *B. coli* grown in a synthetic medium with sulfanilamide and with various concentrations of peptone.

the original inhibitor would not influence the degree of inhibition, whilst a 1000 fold concentration would produce the additional effect. Comparing this experiment with Exp. IV and V it is clear that the action of peptone is much stronger than can be caused by its content of p. aminobenzoic acid.

Experiment IV. *B. coli* was grown in the synthetic medium already mentioned (Exp. I) and the influence of various concentrations of p. aminobenzoic acid was studied. 0.0005 m. mol. p. aminobenzoic acid decreases the sulfanilamide effect. For a total inhibition however 0.005 m. mol. is necessary (fig. 4). As the sulfanilamide concentration is 0.1% or 6 m. mol. the ratio between the concentrations is about 1 : 1200. (for streptococci we found this ratio 1 : 10000, for *Cl. acetobutylicum* RUBBO and GILLESPIE (20) found a ratio 1 : 23000).

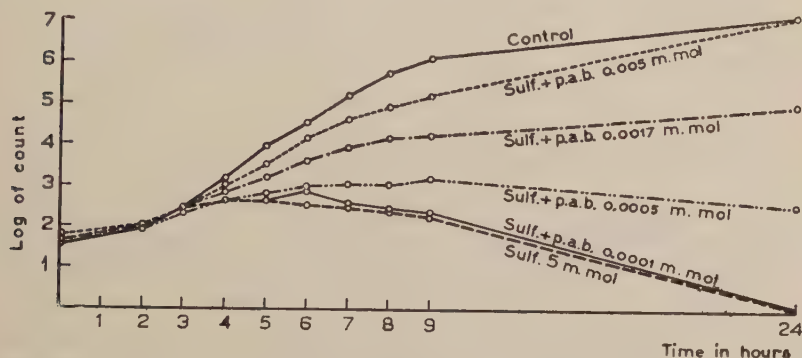


Fig. 4. Growth curves of *B. coli* grown in a synthetic medium with sulfanilamide and with various concentrations of p. aminobenzoic acid.

Experiment V. With the method described in Exp. VII the „concentration” of p. aminobenzoic acid in peptone was estimated. The 10% peptone solution as used in Exp. III gives an extinction which corresponds to 0.05 m. mol. p. aminobenzoic acid (corrections for the extinction of peptone itself were duly made). The major part of the obtained colour is probably due to tryptophane which gives a similar reaction. Calculated as tryptophane the peptone would then contain about 0.1% of this amino acid. So the concentration of p. aminobenzoic acid in the 10% peptone solution is probably much lower than 0.05 m. mol. From Exp. III it is evident that peptone antagonizes sulfanilamide in a concentration of 0.002%, which would correspond with

0.00001 m. mol. p. aminobenzoic acid. In fig. 4 an inhibition of sulfanilamide by p. aminobenzoic acid equal to that of 0.002% peptone is obtained with at least 0.0017 m. mol. p. aminobenzoic acid that is with a concentration over a hundred times the amount possibly present in the peptone. Thus the sulfanilamide inhibition by peptone cannot be due to p. aminobenzoic acid!

Experiment VI. *Streptococcus haemolyticus* was cultured in a medium containing 2.5 ml horse blood, 1.5 ml infusion broth (with 1% peptone) and 1 ml distilled water or added substances.

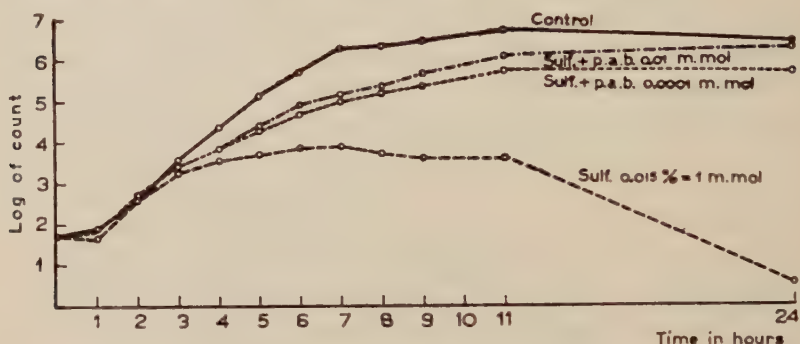


Fig. 5. Growth curves of *Streptococcus haemolyticus* grown in a medium containing 2.5 ml horse blood, 1.5 ml infusin broth, 0.015% sulfanilamide and with p. aminobenzoic acid.

The action of sulfanilamide and p. aminobenzoic acid was studied. The resulting growth curves are reproduced in fig. 5. In this medium containing 0.3% peptone, sulfanilamide is active (see previous publications in this series). Still 0.0001 m. mol. p. aminobenzoic acid is counteracting the sulfanilamide (ratio 1 : 10000). If 0.3% peptone actually contained (see Exp. V) $0.03 \times 0.05 = 0.0015$ m. mol. p. aminobenzoic acid the fifteen times lower concentration of added p. aminobenzoic acid could not possibly show a counteracting effect.

So again we must conclude that the concentration of p. aminobenzoic acid in peptone (and in the blood for that matter) must be so low as to be negligible. The sulfanilamide inhibition by peptone cannot be due to p. aminobenzoic acid. With *B. coli* cultured in peptone water similar observations are made and the same reasoning holds true.

Experiment VII. Sulfanilamide and p. aminobenzoic acid can be estimated quantitatively by diazotization and coupling f.i. to

oxychinoline and colorimetric determination of the resulting diazocompound with the aid of a Pulfrich Stufenphotometer (Zeiss).

To 1 ml of a solution of p. aminobenzoic acid were added 1 ml 1% HCl, 1 ml 0.3% NaNO₂ and 1 ml of a 0.1% solution of oxychinoline in 1% HCl. The mixture was kept for 10 minutes at 0° C. 1 ml 2 N NaOH was now added and the mixture left for 10 minutes at room temperature. After adding distilled water up to a volume of 50 ml the intensity of the bright orange colour of the diazocompound was determined in an 1 cm layer in a Pulfrich Stufenphotometer with a filter S 50. With standard solutions extinction/concentration curves for p. aminobenzoic acid and sulfanilamide were obtained. With this method the adsorption of sulfanilamide and p. aminobenzoic acid to *B. coli* was determined (Exp. A and B).

B. coli was grown in 1000 ml glucose broth for 16 hours. The bacteria were centrifuged down, washed once with distilled water and finally suspended in 8 ml distilled water. The concentration of these suspensions was found by centrifuging a sample in capillary tubes at 10000 rpm. for 20 min. and is given as % by volume (12.7% corresponds with about 4×10^{10} viable bacteria per ml).

2 ml of a solution of p. aminobenzoic acid or sulfanilamide were added to 2 ml bacterial suspension or to 2 ml distilled water. After centrifuging the concentration of p. aminobenzoic acid or sulfanilamide in the supernate was determined. In the case of sulfanilamide the concentrations in the supernate and in the control always were equal (table I). This means that the bacteria neither adsorb nor exclude the drug, which seems to permeate freely (with impermeable bacteria a difference in the concentration of about + 10% was to be expected; for in the control 2 ml solution of sulfanilamide are diluted with 2 ml distilled water, but in the case of a 20% suspension of (impermeable) bacteria it would be diluted with 1.6 ml water only).

Exp. A. Adsorption of sulfanilamide by *B. coli*.
2 ml sulfanilamide solution were added to 2 ml bacterial suspension

Table I

concentration of sulfanilamide in m. mol. per litre						
added.	39	7.8	1.45	0.92	0.46	0.23
found a) water	19.4	3.9	0.72	0.46	0.23	0.13
b) suspension	20	3.9	0.72	0.46	0.23	0.13

(19.2%) or to 2 ml water respectively. After centrifuging the concentrations of sulfanilamide in the supernate were determined (table I).

Similar experiments with red blood cells (horse and sheep) or liver cells (mouse) rendered it equally certain that in neither case sulfanilamide was adsorbed by or excluded from the cells.

Exp. B. Adsorption of p. aminobenzoic acid by *B. coli*.

2 ml p. aminobenzoic acid solution were added to 2 ml bacterial suspension (12.7%) or to 2 ml water respectively. After centrifuging the concentrations of p. aminobenzoic acid in the supernate were determined (table II).

Table II

concentration of p. amino- benzoic acid in m. mol. per litre					
added	25	5	1	0.5	0.1
found a) water	12.5	2.5	0.5	0.25	0.05
b) suspension	10.2	1.92	0.425	0.18	0.04
Δ =adsorbed quantity (a-b)	2.3	0.58	0.075	0.07	0.01
log Δ	0.37	0.76 -1	0.88 -2	0.84 -2	0.0 -2

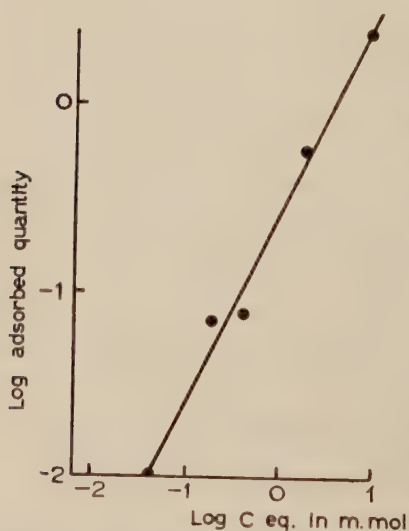


Fig. 6. Adsorption of p. aminobenzoic acid by *B. coli*.

This experiment makes it evident that p. aminobenzoic acid is strongly adsorbed by *B. coli*. The adsorption follows FREUNDLICH'S adsorption isotherm (fig. 6).

Exp. C. Adsorption of p. aminobenzoic acid by *B. coli* heated in a water bath at 60° C.

2 ml p. aminobenzoic acid solution were added to 2 ml bacterial suspension (12.7%) or to 2 ml water respectively. After heating and centrifuging the concentrations of p. aminobenzoic acid in the supernate were determined (table III).

Table III

time of heating in minutes	0	15	30	60	120	180
concentration of p. aminoben- zoic acid in m. mol. per litre						
found a) water	0.64	—	—	—	—	—
b) suspension	0.49	0.625	0.64	0.64	0.64	0.64
log of viable count per ml . .	10.63	9.89	8.92	6.45	3.22	2.14

As soon as the viable count drops below 10% of its original value the adsorbed quantity comes within the experimental error. Dead bacteria do not adsorb p. aminobenzoic acid.

No adsorption of p. aminobenzoic acid could be observed with heated bacteria in this experiment.

Red blood cells and liver cells do not adsorb p. aminobenzoic acid. So it seems to be a special property of the living bacterial cells.

Summary.

The theory of KUNN and others, who explain the action of sulfanilamide and its antagonist p. aminobenzoic acid, by supposing the latter substance to be the prosthetic grouping of a bacterial enzyme, is discussed. Various facts which are not in accordance with this hypothesis are considered. It is emphasized that the growth promoting property and the sulfanilamide antagonizing property of p. aminobenzoic acid need not necessarily be correlated. If p. aminobenzoic acid counteracts sulfanilamide this does not necessarily mean that the sulfanilamide acts by counteracting p. aminobenzoic acid as for many kinds of bacteria it is by no means proven that p. aminobenzoic acid is a growth factor.

In connection with the discussion experiments are published to show that:

1. *B. coli* grows in synthetic media without p. aminobenzoic acid. Still sulfanilamide inhibits growth in this medium and p. aminobenzoic acid is antagonistic (Exp. I and IV).

2. Cysteine is antagonistic to sulfanilamide with streptococci (Exp. II).

3. Peptone is strongly antagonistic for sulfanilamide (Exp. III). This action is not due to p. aminobenzoic acid.

4. p. Aminobenzoic acid is strongly adsorbed by *B. coli*. This ad-

sorption cannot be due to the apoferment. Sulfanilamide is either not adsorbed or if it is, adsorption is within the experimental error.

It is still unknown which part of bacterial metabolism sulfanilamide interferes with, by supplanting p. aminobenzoic acid or not. Various considerations led us to the assumption that sulfanilamide interferes with protein metabolism. Within this scope some alternative hypotheses about sulfanilamide action are offered.

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(From the Biophysical Research Group Utrecht-Delft under the Direction of A. J. KLUYVER, Delft).

SOME OBSERVATIONS ON THE UTILIZATION OF ORGANIC COMPOUNDS BY PURPLE SULPHUR BACTERIA

by

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1. INTRODUCTION.

In recent years purple sulphur bacteria have mostly been cultivated in media containing considerable quantities of organic substances. Especially salts of fatty acids have been found to be suitable in the promotion of the development of these bacteria.

This observation is remarkable in view of the conceptions of the earlier investigators regarding the metabolism of these bacteria. The early development of these conceptions is closely connected with the history of the discovery of the chemo-autotrophic organisms, which has recently been outlined by KLUYVER (2).

In later years more especially the classical investigations of WINOGRADSKY have often been interpreted as pointing to a strictly autotrophic nature of the purple sulphur bacteria. However, this interpretation does not appear to be fully justified, as will be expounded below.

In 1887 WINOGRADSKY (7) published his well-known study on the metabolism of the colourless sulphur bacteria. Here he arrives at the conclusion that the oxidation of sulphur compounds is the only source of energy for these organisms (p. 592). Owing to this process they can thrive upon very small amounts of organic substances, since these substances are not consumed in respiration. Moreover these bacteria would not have special demands regarding the nature of the organic substrates. This fact, according to WINOGRADSKY, would be due to

¹⁾ 11th Communication: *Enzymologia* **10**, 355, 1942.

the circumstance that a decomposition of the organic substances is not required for energy supply. WINOGRADSKY mentions explicitly that the sulphur bacteria are able to utilize fatty acids. As to a possible utilization of carbon dioxide for assimilatory purposes no indications whatever are found in the mentioned paper.

In 1888 WINOGRADSKY (8) published his fundamental investigations concerning the metabolism of purple sulphur bacteria. They were found to have many features in common with the colourless sulphur bacteria, *e.g.*, they also oxidize hydrogen sulphide and develop in media with very low concentrations of organic substances. They were found to differ, however, by their marked sensitivity towards oxygen, from which gas only low pressures were endured. The favourable influence of light on the development of purple sulphur bacteria was interpreted as being due to the production of small amounts of oxygen in the light by green bacteria, which as a rule accompany the purple bacteria in nature. The photosynthetic function of the purple sulphur bacteria, as advocated by ENGELMANN, was not accepted by WINOGRADSKY (p. 51—56). In this paper too any definite suggestion regarding an autotrophic metabolism of the purple sulphur bacteria is lacking.

In 1890 WINOGRADSKY (9) published a study on nitrifying bacteria, and quoted the opinion of HUEPPE that these bacteria exert a „Chlorophyllwirkung ohne Chlorophyll“, which then would imply that carbon dioxide is split, and that the liberated oxygen oxidizes the ammonia. WINOGRADSKY concludes that, if this would hold, nitrification should also proceed in the absence of air, and since this does not occur he rejects HUEPPE's theory.

However, in the same paper WINOGRADSKY himself actually proves that the nitrifying organism is able to assimilate carbon dioxide. Concerning the metabolism he concludes: „En rejetant pour ces raisons l'hypothèse de l'action chlorophyllienne sans chlorophylle, une autre semble s'imposer: c'est celle de la synthèse d'un amide quelconque au dépens de l'acide carbonique et de l'ammoniaque" (p. 274). The nitrifying organism proved to have a number of remarkable qualities, *viz.*, 1) their synthetic power prevails, so that an accumulation of organic substances results. „En cela il est comparable aux plantes à chlorophylle"; 2) decomposition processes of organic matter are either absent or occur in a very restricted measure only. They are replaced by the oxidation of ammonia, which process furnishes all energy necessary for the vital activity of the cells.

It is clear that the nitrifying organisms studied represent a quite

remarkable physiological type and it is suggested (p. 275) that both the sulphur oxidizing and the iron oxidizing organisms may also belong to the same type. Thus here for the first time the type of metabolism, later on designated as chemo-synthetic or chemo-autotrophic, has been extended to the sulphur bacteria as well. We may take it for granted that both the colourless and the purple forms are included in this statement. From the foregoing it is evident that the conclusion as to the strictly chemo-autotrophic character of the sulphur bacteria was reached by WINOGRADSKY only by analogy.

SKENE (5) working with crude cultures of purple sulphur bacteria arrived at the conclusion that their growth was influenced not at all favourably, in fact rather inhibited, by addition of organic substances to the medium. His conception of the metabolism of the purple sulphur bacteria is very similar to that of WINOGRADSKY. He emphasized, however, that both the presence of hydrogen sulphide, and the accessibility of light are necessary for the development.

Also BUDER (1) lays stress upon the available evidence that the purple sulphur bacteria „benötigen für eine üppige Entfaltung organische Substanz, wenn überhaupt, so doch höchstens in ganz geringer Menge" (p. 532), and on the whole endorses the views of WINOGRADSKY and of SKENE. BUDER concludes that „eine kritische Sichtung der bisherigen Erfahrungen legt vielmehr den Schluss ausserordentlich nahe, dass sie autotroph sind" (p. 532).

VAN NIEL (4) in his well-known studies of the purple sulphur bacteria, has interpreted WINOGRADSKY's views as implicating the autotrophic or chemosynthetic character of the purple sulphur bacteria. VAN NIEL himself cultivated pure strains in completely inorganic media and established the stoichiometrical relation which exists between the amounts of CO_2 and of H_2S that disappear. At about the same time, however, he observed that for his strains various organic substances could replace the sulphide, and he concludes „that of the comparatively large number of pure cultures investigated thus far not a single one behaves as an obligate autotrophant". VAN NIEL then expresses the view that all organisms belonging to the *Thiorhodaceae* can utilize organic matter, although they are photosynthetic autotrophants (p. 102). It can be added that shortly afterwards MULLER (3) has studied in greater detail metabolic reactions of *Thiorhodaceae* with organic substrates.

The situation outlined above, viz., on the one hand the prevailing tendency of ascribing a fully autotrophic character to the *Thiorhodaceae*, and on the other hand the observations regarding the favourable effect

of the addition of organic substances to the culture media, forms the starting point for the present investigation.

We have, therefore, attempted to isolate a strain of *Thiorhodaceae* which would be indifferent to the addition of organic substances, *i.e.*, a strain the metabolism of which would closely answer the conception made of the metabolism of purple sulphur bacteria in the earlier literature.

2. ENRICHMENT CULTURES.

Enrichment cultures were made in the medium prescribed by VAN NIEL ((4), p. 11), to wit: 0.1% NH_4Cl , 0.05% K_2HPO_4 , 0.02% MgCl_2 with varying amounts of NaHCO_3 and Na_2S (cf. Table I).

Glass-stoppered bottles of 100 cc were used; they were inoculated with mud from a ditch at Delft, and incubated in a light cabinet, at about 30° C.

A set of 36 cultures was started simultaneously in media which contained various amounts of bicarbonate and sulphide, whereas also the pH was varied. It has to be taken into account, however, that the pH can not be varied independently of the variation of bicarbonate and sulphide. Therefore, the given figures only indicate the amounts of the mentioned compounds initially added. Variations in pH were established either by addition of 10% H_3PO_4 or of 10% Na_2CO_3 .

The following variables were introduced: NaHCO_3 0.5%, 0.2%; Na_2S 0.2%, 0.1%, 0.05%; pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.5. A survey of the composition of the media in the 36 bottles is given in Table I.

Table I

Variations in the composition of the culture medium in the 36 bottles used for enrichment cultures.

NaHCO_3	0.5%	0.2%	0.5%	0.2%	0.5%	0.2%
Na_2S	pH 7.0		pH 7.5		pH 8.0	
0.2 %	No 1	No 2	No 7	No 8	No 13	No 14
0.1 %	.. 3	.. 4	.. 9	.. 10	.. 15	.. 16
0.05%	.. 5	.. 6	.. 11	.. 12	.. 17	.. 18
0.2 %	No 19	No 20	No 25	No 26	No 31	No 32
0.1 %	.. 21	.. 22	.. 27	.. 28	.. 33	.. 34
0.05%	.. 23	.. 24	.. 29	.. 30	.. 35	.. 36
	pH 8.5		pH 9.0		pH 9.5	

Concerning the development of the cultures the following observations can be recorded:

- No 1. Eleven days after inoculation there was an abundant growth of green bacteria, which were overgrown by purple bacteria after 20 days.
- No 2. Development of green bacteria was observed after 6 days, after 13 days the culture was overgrown by purple bacteria.
- No 3. Development identical with No 1.
- No 4. Development identical with No 2.
- No 5. After 7 days green bacteria had developed, after 20 days purple ones as well.
- No 6. After 7 days green bacteria, the culture was very homogeneous; not before 30 days after inoculation purple bacteria appeared.
- No 7. After 11 days few green bacteria, after 28 days only purple bacteria in a fairly thick layer of deep red colour.
- No 8. No development of green bacteria occurred, there was an abundant growth of a rather large *Chromatium* species¹).
- No 9. After 11 days a slight development of green bacteria, after 28 days only a slight development of purple bacteria.
- No 10. After 13 days purple bacteria belonging to a small *Chromatium* species; no green bacteria were observed.
- No 11. Development practically identical with No 9.
- No 12. Development practically identical with No 9.
- No 13. After 13 days a very slight development of green bacteria; after 15 days the culture was already overgrown by purple bacteria, which developed merely slightly.
- No 14. After 6 days abundant growth of purple bacteria of brownish red colour.
- No 15. After 8 days growth of purple bacteria of bright red-brown colour.
- No 16. After 6 days a layer of purple bacteria of an orange-red colour.
- No 17. After 8 days abundant growth of lilac-red bacteria.
- No 18. After 7 days purple bacteria of pink colour.
- No 19. After 27 days slight development of purple bacteria.
- No 20. After 6 days development of a red-brown culture.
- No 21. After 17 days development of bacteria of a lilac-red colour.
- No 22. Slight development of purple bacteria starting after 15 days.
- No 23. Slight development of purple bacteria starting after 13 days.
- No 24. After 11 days development of purple bacteria of bright red colour.
- No 25. No development was observed within three months.
- No 26. After 40 days a pale-red layer of bacteria had grown.
- No 27—30. No development was ever observed.
- No 31—36. No development was ever observed.

We may conclude from these observations, that development of photosynthetic autotrophic bacteria occurs between pH 7 and pH 9.

¹) cf. A. MANTEN, this Journal, p. 164.

At a pH of 7.0 to 7.5 chiefly green bacteria developed, which, however, later on were overgrown by purple bacteria; at a higher pH exclusively purple bacteria developed.

In general the results are in agreement with those of VAN NIEL. In detail some differences were observed, *e.g.*, VAN NIEL reports that at pH 9.0 more cultures developed, while development at pH 7.0 was not observed in his experiments.

3. THE PURIFYING PROCEDURE.

Some cell material of most of the better developed enrichment cultures was transferred to bottles containing the corresponding media. After fresh development had occurred, material of the latter cultures was transferred to media, the composition of which was slightly different as to its Na_2S and NaHCO_3 content. It was found that these subcultures in many cases showed a more abundant growth.

From these subcultures agar shake cultures were made in order to obtain pure cultures in the way described by VAN NIEL.

Briefly this method is as follows. Agar containing 0.1% NH_4Cl , 0.05% K_2HPO_4 and 0.02% MgCl_2 in tap water is boiled in order to remove oxygen and then cooled down to 60° C.; at this temperature NaHCO_3 and Na_2S are added under aseptic conditions and the pH is adjusted at the desired value. Culture tubes are filled and cooled down to 45° C. One culture tube is inoculated with some drops of bacterial suspension and then shaken in order to disperse the suspension evenly in the medium. From this tube some drops are brought into a second one, and so on, till 6 or 7 dilutions are obtained. The tubes are quickly cooled, and then kept free from oxygen by means of a cotton plug with pyrogallol and KOH, and incubated in a light cabinet at about 30° C.

In the highest dilutions separate colonies developed within a few days; from such colonies transfers were made into another set of tubes. Sometimes either no development had taken place, or the cultures were too crowded. In such cases the procedure had to be made over again.

Altogether 10 pure cultures have been obtained, isolated from the standard medium in the following variations:

No 7a	containing	0.5% NaHCO_3 and 0.2% Na_2S ; pH 7.5.
No 8c	„	0.2% NaHCO_3 and 0.1% Na_2S ; pH 7.5.
No 10b	„	0.5% NaHCO_3 and 0.1% Na_2S ; pH 7.5.
No 15b	„	0.2% NaHCO_3 and 0.1% Na_2S ; pH 8.0.
No 16b	„	0.5% NaHCO_3 and 0.1% Na_2S ; pH 8.0.
No 17a	„	0.5% NaHCO_3 and 0.05% Na_2S ; pH 8.0.
No 18c	„	0.2% NaHCO_3 and 0.1% Na_2S ; pH 8.0.

No 20 containing 0.5% NaHCO_3 and 0.2% Na_2S ; pH 8.5.

No 21b „ 0.2% NaHCO_3 and 0.1% Na_2S ; pH 8.5.

No 24b „ 0.2% NaHCO_3 and 0.1% Na_2S ; pH 8.5.

Special notice was taken of the fact that in most of the cases the pure cultures had the same colour as the corresponding enrichment cultures, so that it is evident that in those cases the organism prevailing in the enrichment culture had actually been isolated.

Concerning the morphology of the isolated strains we remark that the same three types as mentioned by VAN NIEL were found, *i.e.*, the *Chromatium* type, the *Thiocystis* type and the *Pseudomonas* type. No 8c and No 10b were of the *Chromatium* type, No 17a was of the *Thiocystis* type, whereas the remaining strains all belonged to the *Pseudomonas* type.

In connection with the observations on the infrared absorption spectra of purple bacteria, made by WASSINK, KATZ and DORRESTEIN (6), we were also interested in the infrared absorption spectra of our newly isolated strains. Only the strains which showed an abundant growth were examined, while the other isolated strains had developed to such a slight degree, that it was not possible to gain enough material for the measuring of absorption spectra.

In Fig. 1 some of the spectra are represented; strain D — the strain

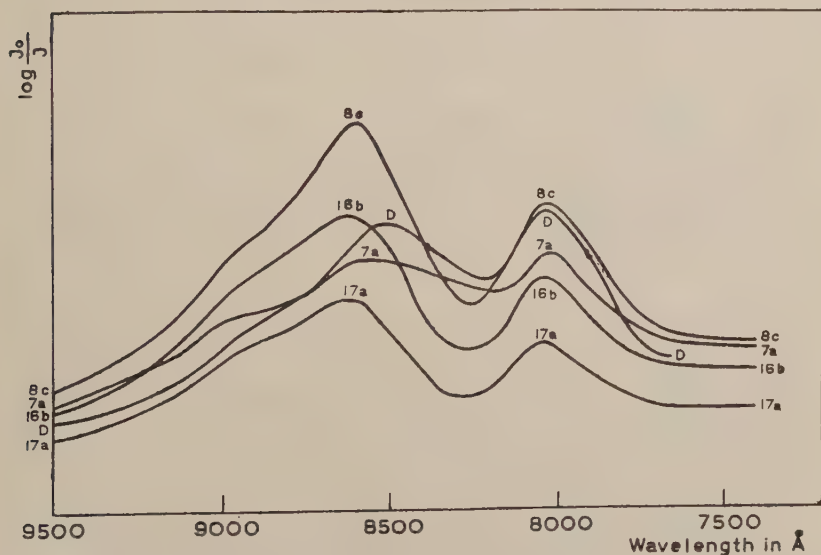


Fig. 1. Infrared absorption spectra of some newly isolated strains of purple sulphur bacteria.

normally used in our investigations — is given for the sake of comparison.

All the spectra show the same type, with two distinct maxima between 8000 and 9000 Å. It is remarkable that both maxima show variations in situation in the various strains, whilst these variations are not correlated; thus the distance between the maxima is not the same in the different strains. This result substantiates the conclusion, previously drawn by WASSINK, KATZ and DORRESTEIN (6), that the various maxima represent separate pigment-protein complexes.

It may be remarked here, that the strains D and 8c belong to the *Chromatium* type, strains 7a and 16b to the *Pseudomonas* type, whereas strain 17a is a representative of the *Thiocystis* type. It appears, therefore, that the situation of the maxima of the absorption bands is not correlated with the morphological type of the cells. So e.g. in the *Pseudomonas* type 16b and in the *Thiocystis* type 17a the situation of the maxima is the same, whereas the two *Chromatium* strains D and 8c differ in the situation of one of their maxima.

4. THE UTILIZATION OF ORGANIC SUBSTANCES.

As has been put forward in the introduction, it was our object to isolate, if possible, a strain indifferent towards organic substances. In order to test the isolated strains with regard to this quality, they were all cultivated in media containing an organic substrate.

Each of the strains was inoculated into a stoppered bottle; after development had taken place, the culture was shaken in order to obtain a homogeneous suspension. From this suspension 5 cc were inoculated both into a stoppered bottle of 50 cc with the corresponding inorganic medium and into a stoppered bottle of 50 cc with the same medium to which 0.2% Na-malate had been added.

Without exception all strains tested in this way showed a markedly stronger and faster development in the medium to which malate had been added. Within three or four days in the organic medium a dense suspension of a deep purple colour was obtained, contrary to what happened in the inorganic cultures. These showed a weaker development and attained their maximum after at least a week. Although all the strains had been isolated from a strictly inorganic medium, all of them grew much faster in organic media. Obviously the photo-heterotrophic mode of life is much more favourable to these bacteria than the photo-autotrophic one.

It was found, that the ordinary malate-thiosulphate medium previously found to be optimal for strain D (inorganic salts with 0.2% Na-malate and 0.1% thiosulphate with a trace of Na_2S) was also most suitable for the newly isolated strains, on condition that the pH corresponded to that of the inorganic medium from which the strain had been isolated. In order to keep the pure cultures it was, therefore, not necessary to prepare different media, as it was possible to use one medium at different pH, which means a considerable simplification in the preparation of the media.

Thus, all strains which had been isolated from a strictly inorganic medium grew much faster in organic media. We have not succeeded in isolating a single obligately autotrophic strain of purple sulphur bacteria. It is evident that in general the heterotrophic mode of life is more favourable to purple sulphur bacteria than the autotrophic one, and that the autotrophic character of these bacteria has been overestimated, especially in the earlier literature. The experiments made leave no doubt that the enrichment procedure as devised by VAN NIEL, applied to ordinary mud, yields chiefly forms appreciative of organic nutrients. It remains, however, desirable to investigate whether forms, adapted to life in special media, such as *e.g.* sulphur springs, might behave otherwise.

Summary.

Enrichment cultures of purple sulphur bacteria from Delft mud were made in inorganic media. By means of shake-cultures in agar with inorganic salts it was attempted to isolate a strain, the growth of which was not favoured by organic substances. This aim could not be attained as all strains isolated thrived better in media containing sodium malate than in completely inorganic ones. So it may be concluded that purple sulphur bacteria, at least those isolated from ordinary mud, show a better development under photo-heterotrophic than under photo-autotrophic conditions.

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THE ISOLATION OF *CHROMATIUM OKENII* AND ITS BEHAVIOUR IN DIFFERENT MEDIA

by

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1. INTRODUCTION.

During an investigation concerning the possible existence of obligately autotrophic *Thiorhodaceae* (4), an enrichment culture was obtained in which a motile species of *Chromatium* with remarkably large cells (8—10 μ long) prevailed. According to the diagnoses of *Chromatium* species, to be found in literature (cf. e.g. ELLIS (1) and WINOGRADSKY (5)), the cells in our culture appeared to belong to the species *Chr. Okenii*. Since pure cultures of this species have not yet been described, isolation was attempted at. In pure culture the strain showed a marked pleomorphism which was studied in some detail.

2. EXPERIMENTAL.

The enrichment culture was obtained as follows.

A stoppered bottle was filled with a mineral solution of the following composition: Delft tap water to which 0.1% NH_4Cl , 0.05% K_2HPO_4 , 0.02% MgCl_2 , 0.2% NaHCO_3 , and 0.2% Na_2S were added, pH was adjusted to 7.5 by addition of H_3PO_4 . The bottle was completely filled with the culture medium, inoculated with about 1 cc mud of a Delft ditch; the stopper was applied with careful exclusion of all air, and the bottle exposed in a light cabinet at 25—30° C. After a week a dark red enrichment culture of strongly motile bacteria of 8—12 μ length had developed. After transferring material from this enrichment culture into fresh bottles with the same medium cultures of small bacteria, of only 3—4 μ length, were obtained. The absence of mud appeared to influence unfavourably the size of the cells.

The bacteria were brought into pure culture, following the prescriptions given by VAN NIEL (2), i.e. by shaking them in melted agar containing the same nutrients as the enrichment medium, and making dilutions.

After 10 days exposure in the light cabinet, separate colonies of purple sulphur bacteria had developed in the highest dilutions. By inoculating

part of these colonies in stoppered bottles with the culture medium, none of the cultures obtained turned out to be pure. All bottles contained next to the *Chromatium* small rod-shaped purple sulphur bacteria. Neither did shaking a second time in melted agar bring about any improvement; therefore shake cultures were made for a third time, now in malate-thiosulphate agar (with 0.2% Na-malate and 0.1% Na-thiosulphate instead of NaHCO_3 and Na_2S). This time two types of large colonies developed, which distinctly differed in colour, viz., markedly blackish-red morello-coloured colonies of *Chr. Okenii* about 2 mm in diameter, and pinkish-coloured somewhat smaller colonies of the contaminating form. Some of the dark red colonies appeared to be pure as judged from microscopic examination. Without suspending it in a liquid medium, material of the pure colonies was once more divided into an agar medium, and this process was still repeated twice. Now in the highest dilutions exclusively colonies of *Chr. Okenii* developed. Here upon the culture was considered to be pure.

It was not possible to cultivate the contaminating form alone. Indeed next to the blackish-red colonies of *Chr. Okenii*, colonies developed which contained merely small rod-shaped bacteria; after transferring these colonies, however, no development was observed.

The size of the bacteria of the dark red colonies in malate-thiosulphate agar was merely about $5\ \mu$, thus far lower than the size of *Chr. Okenii* in the enrichment culture. After transferring into a liquid medium with malate-thiosulphate solution, at first cells were obtained of about the same size as in the corresponding agar. Afterwards a strong differentiation in cell size occurred: on the one hand bacteria of about $15\ \mu$ length developed, and on the other hand bacteria of merely about $1\ \mu$. Between these limits all transitions in size were present.

The bacteria from $3-6\ \mu$ as a rule were motile, whereas the smaller and the larger forms only occasionally were seen moving. The very large forms ($10-15\ \mu$) had a marked red colour, even visible in a single cell at 1000 times magnification. The size of these cells agreed with that given by WINOGRADSKY (5) for *Chr. Okenii*. They either did not move at all or only rarely, and they never contained any sulphur drops. Their form was mostly irregularly round and seldom cylindrical. Altogether they did not appear very healthy. The middle-sized forms were much more motile, cylindrical or vibrio-shaped, and they too had little or no sulphur drops in their cells.

The following part of the investigation aimed at the finding of a medium in which none but normal forms of large size would develop.

For this purpose a series of 16 bottles containing the mineral culture medium with Na_2S and NaHCO_3 , pH 7.5, to which different amounts of Na-malate and Na-thiosulphate had been added, was inoculated with the pure strain, and incubated in the light cabinet. After an exposure of about 4 weeks a marked differentiation in the appearance of the bacteria in the different media was observed. The bottles were labeled as indicated in the following table, in which some characteristics of the cultures have been given, such as were apparent at the 30th day after inoculation.

At the moment of observation, in the set of bottles with 0.1% Na-malate the conditions apparently favoured the appearance of strongly motile forms (Nos 6, 10, and 14).

Table I

Development of *Chr. Okenii* in culture media of different composition.

Na- thiosulphate \ Na-malate	0.05%	0.1%	0.2%	0.4%
0.05%	1) S: — M: — B: —	2) S: — M: — B: —	3) S: ± M: ± B: ±	4) S: — M: ± B: ±
	5) S: — M: — B: —	6) S: ± M: + B: +	7) S: ± M: ± B: +	8) S: ± M: ± B: ±
	9) S: ± M: — B: —	10) S: + M: ++ B: +	11) S: + M: + B: +	12) S: ± M: ± B: +
0.1%	13) S: ++ M: + B: ++	14) S: ++ M: ++ B: ++	15) S: + M: + B: +	16) S: ± M: ± B: +
0.2%				
0.4%				

S = presence of sulphur drops in the cells

— : absent

M = motility of the bacteria

± : scanty

B = occurrence of large-sized bacteria ($> 6 \mu$)

+ : moderate

++ : strong

Especially the culture No 14 and also No 13 were very satisfactory in so far as the high thiosulphate concentration in combination with a low malate concentration gave rise to a quite homogeneous population in which all bacteria were about 8μ long and 3.5μ wide, whilst nearly half of the cells were strongly motile. The bacteria were filled with large, highly refractive sulphur drops, microscopically they appeared very similar to those of a normal enrichment culture of *Chr. Okenii*; merely their average size was somewhat smaller. The presence of the sulphur drops in the bacteria apparently caused the pink colour of the bottles contrary the dark red colour of the bottles which had been supplied with less thiosulphate.

Anyhow, these observations leave no doubt as to the possibility to obtain large cells again from colonies containing none but small forms, by variation of the culture-medium.

3. DISCUSSION.

Several investigators have tried to isolate *Chr. Okenii* without success. VAN NIEL (2), who was the first to obtain pure cultures of purple sulphur bacteria, also reports unsuccessful attempts. In one case VAN NIEL could note that a shake culture of *Chr. Okenii* in agar only showed proliferation when a heavy inoculation containing green bacteria was used. In another case he observed that an isolated colony developed, which was in contact with a small air-canal (*l.c.* p. 25). The favourable influence of the presence of green bacteria and apparently in some cases also of a small oxygen supply upon the development of large-sized *Chromatium* species was also observed already by WINOGRADSKY (5). According to VAN NIEL these various observations may possibly be understood from the viewpoint that the mentioned circumstances would locally give rise to a tension of H_2S , favourable for the development of the large-sized *Chromatium* (*l.c.* p. 47). After this conclusion was reached, VAN NIEL apparently has made no further attempts to isolate species of this group.

According to an oral communication of Prof. KLUYVER it has also been observed several times in the Delft laboratory that from inoculations with large-sized bacteria only small forms resulted which then were discarded as lacking the characteristics of *Chr. Okenii*. These small forms, according to the observations discussed in the present paper, may at least partly have actually belonged to *Chr. Okenii*, developing under sub-optimal conditions.

WINOGRADSKY (5) distinguished 5 species of *Chromatium*, according to the size of the cells closely before and after cell division; for *Chr. Okenii* a length of 7.5–15 μ was given.

Our observation that in agar with 0.2% malate and 0.1% thiosulphate the size of the cells of *Chr. Okenii* was found to vary from 1–15 μ , makes it evident that caution is needed for characterization of species of *Chromatium* according to their size without simultaneous indication of the cultural conditions.

On the other hand it remains questionable whether it is allowed to consider *Chr. Weissii*, *Chr. vinosum*, *Chr. minus* and *Chr. minutissimum* merely as forms of *Chr. Okenii*, as has been advocated by ELLIS (1). For example the species of *Chromatium*, designated by ROELOFSEN (3) as „strain D”, which answers best to the diagnosis of *Chromatium minus* markedly differs in behaviour from *Chr. Okenii* and does not develop large cells when cultivated in the medium found optimal for *Chr. Okenii*. Besides this mention may be made of our observation that the situation of the infrared absorption maxima in suspensions of strain D, differs from that of the strain of *Chromatium Okenii* isolated (strain 8c (4)).

From the experiments, discussed in the present paper, it is clear that

the species studied, which under special conditions satisfactorily meets the diagnosis of *Chr. Okenii*, shows strong variations in size, dependent on the culture conditions. On the other hand it appears from the foregoing that various strains of *Chromatium* isolated so far, show cells of markedly different size under identical conditions, and thus in a taxonomy based mainly on the size of the cells, they would have to be considered as different species.

Finally it may be mentioned that the development of *Chr. Okenii* in its most characteristic forms in the media Nos 13 and 14 supports the ideas of WINOGRADSKY concerning the most suitable conditions for development of purple sulphur bacteria in nature, viz., sulphur compounds in high concentrations, and a small amount of organic substances. On the other hand, however, it must not be overlooked that the rate of development of all species examined in pure culture, is strongly increased by the supply of larger amounts of organic nutrients (cf. (4)).

S u m m a r y.

An enrichment culture of *Chromatium Okenii*, obtained from mud of a ditch at Delft, was used for isolation of this species. The bacteria of the strain, isolated by making 5 successive shake cultures in an agar medium were, however, less than half the size of *Chr. Okenii* when cultivated under the same conditions as the bacteria in the original culture.

By variation of the culture conditions for the pure strain, it appeared that in a mineral medium containing fairly high concentrations of sodium thiosulphate and fairly low concentrations of sodium malate, bacteria developed of nearly the same size as present in the enrichment culture. This supports the view of WINOGRADSKY that media of this type offer the most natural conditions to purple sulphur bacteria.

It is, therefore, evident that *Chr. Okenii* is a highly pleomorphic species, and the consequences hereof for the differentiation of species within the genus *Chromatium* were discussed.

I am greatly indebted to Dr E. C. WASSINK, under whose supervision this study was made, for helpful criticism.

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